THE UNIVERSITY
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Monitoring human enterovirus prevalence in Edinburgh by PCR amplification of local sewage

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Declaration

I hereby declare that this dissertation is of my own composition, and that it contains no material previously submitted for the award of any other degree. The work reported in this thesis has been executed by myself, except where due acknowledgement is made in the text.

…….
Julius Owusu Paddy
(30th November 2015)

Acknowledgement

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Furthermore, I would like to acknowledge the contribution of Nguyen Van Dung, my colleague who contributed enormously to this project by performing viral RNA extraction from many of my sewage samples that were used in this thesis.

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ABSTRACT

ABSTRACT

Enteroviruses are transmitted between individuals mainly via the oral-faecal route and they have been associated with clinical syndromes such as meningitis, encephalitis, myocarditis, common cold, poliomyelitis and many others. *Enterovirus* virions excreted into sewage can remain infectious for several weeks. Circulation of enteroviruses in sewage is a marker of their presence in a particular community and hence sewage monitoring can be a complementary approach to assess their prevalence in a population. Sewage surveillance also offers the additional advantage of identifying circulating enteroviruses that are either subclinical or are yet to be presented clinically. The discovery of wild type 1 poliovirus in Israel in 2013, 11 years after the country was declared Polio-free by the World Health Organisation (W.H.O.) is an example of why sewage surveillance is essential.

Currently, there is limited data on circulating enteroviruses in the Greater Edinburgh area. A six month study was conducted (March 2014 to September 2014) to detect the types of enteroviruses in the sewage of the city through sewage screening, understand their temporal patterns and assess whether these types are clinically significant. Sewage sludge collected every fortnight from the Seafield Sewage Water works, a sewage facility that serves the inhabitants of the city were filtered and concentrated. RNA was eventually extracted from the viral concentrates after which RT-PCR, cloning and sequencing of colonies were performed. Serotypes were identified by sequence BLAST and phylogenetic analysis.

During the study, 31 different serotypes were detected and the most common ones were *Coxsackievirus B5* (CVB5), CVB2, CVA22, CVA4, CVA9, *Echovirus 11* (E11), E7, E30 and E18. *Enterovirus C104*, a rare serotype detected in sewage in June 2014 has been associated with respiratory tract infections in countries like Italy, Switzerland and Japan. Also, CVA6 serotype which was identified in several patients with hand foot and mouth disease in Edinburgh in February 2014 was also detected in sewage collected in March 2014. The detection and characterisation of enteroviruses in sewage is an additional resource to enterovirus surveillance and can help explain enterovirus associated disease trends and warn of possible future outbreaks in the area

CHAPTER 1 - INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Enteroviruses are single stranded positive sense RNA viruses that infect a range of mammalian species including humans. Belonging to the *Picornaviridae* virus family, infection in human beings can result in a wide range of clinical manifestations such as respiratory illnesses (common cold), hand foot and mouth disease, aseptic meningitis, acute flaccid paralysis, neonatal sepsis and many others. Transmitted mainly via the oral-faecal route, they undergo replication mostly in the gastro-intestinal tract. The majority of enterovirus infections in humans beings are either subclinical (asymptomatic) or accompanied by mild symptoms (Mueller *et al*., 2005). Enteroviruses are among the most common viruses that infect human beings (Nix *et al.,* 2006*).*

1.2. CLASSIFICATION HISTORY

Depending on the infectious properties of the virus, such as its pathogenicity in mice, enteroviruses were originally divided into four subgroups, namely echovirus, poliovirus, coxsackie A virus and coxsackie B virus. However, subsequent analysis indicated that the phylogenetic basis for these subgroups in some cases were flimsy especially as different strains of an *Enterovirus* serotype may exhibit different pathogenicities in a mouse system. This consequently confused the subgrouping based on pathogenicity (Hyypia *et al.,* 1997). It was also later realized that there were significant overlaps in the biological properties of viruses in these four groups and this ultimately led to a new classification system of consecutive numbers for more recently isolated viruses such as Enterovirus 71 (Oberste *et al.*, 2002).

1.2.1. Poliovirus: Polioviruses are neurotropic enteroviruses and causal agents of the clinical condition in primates known as paralytic poliomyelitis. The propensity for poliovirus to specifically target the motor neurone upon infection to cause paralysis is actually rare. In fact, only about 1 in 100 cases of infection with poliovirus leads to virus invasion of the central nervous system and replication in the neurone resulting in paralytic poliomyelitis. The types of paralytic poliomyelitis include spinal polio which affects the spine, bulbar polio which affects the brainstem, and bulbospinal polio which affects both the spine and brainstem (Ren and Racaniello, 1992). The discovery and introduction of the inactivated vaccine (Salk *et al*., 1954) and the live attenuated oral vaccine (Sabin, 1957) has proved to be very efficient in drastically reducing the incidence of poliomyelitis across the globe.

1.2.2. Echovirus (ECHO - Enteric Cytopathic Human Orphan): Echoviruses were first isolated from the faeces of asymptomatic children and these viruses produced cytopathic effects in cell cultures. They however failed to cause detectable pathologic lesions in suckling mice despite the cytopathic effect. As they did not show any pathogenic properties in experimental animals, they were named ECHO (Enteric Cytopathogenic Human Orphan) viruses (Hyypia *et al.*, 1997). Initially thought to be an orphan virus, it was later realised that individual serotypes were associated with a wide range of clinical manifestations in human beings such as aseptic meningitis, acute febrile illness and myocarditis. Overall, 34 *Enterovirus* serotypes have been assigned as echoviruses. However, *Echovirus 28* has been reclassified as *Rhinovirus 1* whereas E22 and E23 due to their distinct genetic features have also been re-assigned to the human parechovirus 1 and 2 respectively (Harvala and Simmonds, 2009).

1.2.3. Coxsackieviruses: These are a group of non-polio enteroviruses which have been associated with a range of clinical conditions that lead to severe morbidity and mortality, particularly in infants and children (Tebruegge and Curtis, 2009). Infants infected with *Coxsackievirus* have been shown to be susceptible to diseases like myocarditis, meningitis and encephalitis with a mortality rate as high as 10% (Rhoades *et al*., 2011). *Coxsackievirus A6* for example is also known to be the cause of hand foot and mouth disease as well as myocarditis in infants (Puenpa *et al*., 2013).

In adults, studies have linked *Coxsackievirus* infection during pregnancy to an increase in the rate of spontaneous abortions, foetal myocarditis (Ornoy and Tenenbaum, 2006), and neurodevelopmental delays in new-borns (Euscher *et al.*, 2001). Coxsackieviruses were discovered when suckling mice developed paralysis after being inoculated with faecal suspensions of patients who were suspected of having poliomyelitis but tested negative for poliovirus (Gear, 1984). The symptoms were distinguished from poliomyelitis because the damage responsible for limb paralysis was rather due to widespread lesions in the skeletal muscles and not as a result of virus invasion in the central nervous system as in poliomyelitis. Coxsackieviruses are divided into two groups based on their pathogenicity to suckling mice. Coxsackie A virus causes flaccid paralysis whereas coxsackie B virus causes spastic paralysis (Oberste *et al.*, 2003). Coxsackie A virus currently has about 23 serotypes, and is associated with conditions that affect the skin and mucous membrane like hand foot and mouth disease, herpangina and acute haemorrhagic conjunctivitis. Coxsackie B virus on the other hand currently has 6 serotypes and it is associated with diseases like pleurodynia and pericarditis (Schmidt *et al.*, 1973).

1.3. TAXONOMY OF ENTEROVIRUSES

The advancement in genome science especially sequencing ultimately led to a new method of classifying enteroviruses. Enteroviruses are currently classified based on the virus genomic structure rather than pathogenicity in their hosts.

As at now, the genus *Enterovirus* comprises of hundreds of serotypes which have been distinguished into 12 species namely; *Enterovirus A, Enterovirus B, Enterovirus C, Enterovirus D, Enterovirus E, Enterovirus F, Enterovirus G, Enterovirus H, Enterovirus J, Rhinovirus A, Rhinovirus B and Rhinovirus C.* Only *Enterovirus A - D* and *Rhinovirus A-C* are relevant to human infection and disease. The species of Enteroviruses relevant to human infection and their corresponding serotypes are shown in table 1.3a. As observed from this table, the name of the serotype is not necessarily an indication of what species it belongs to. Serotypes *Coxsackievirus A8* (CVA8), CVA9 and CVA22 for example belong to *Enterovirus A, B* and *C* respectively despite the fact that they are all in the coxsackievirus A subgroup. This is simply because, nomenclature was assigned to the serotypes based on their pathogenicity to suckling mice prior to classifying enteroviruses into species A-D. However, *Echovirus* and *Coxsackievirus B* serotypes all belong to *Enterovirus B.* The three poliovirus serotypes (PV1, 2 and 3), have all been classified into *Enterovirus C.*

Species of rhinoviruses on the other hand are the predominant causal agents of common cold. Rhinoviruses were originally classified as a different taxonomical genus also belonging to the Picornaviridae family. However, in 2005 Rhinovirus

species A, B and C were assigned to the *Enterovirus* genus by the International Committee on the Taxonomy of Enteroviruses (Stanway *et al*., 2005).

Table 1.3a; table showing the various species of enteroviruses and their corresponding types.

ENTEROVIRUS SPECIES	SEROTYPES
Enterovirus A	Coxsackievirus A2, A3, A4, A5, A6, A7, A8, A10, A12, A14, A16. Enterovirus A71, A76, A89, A90, A91, A114, A119, A120
Enterovirus B	Echovirus 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 30 Enterovirus B69, 73, 74, 75, 77, 78. B82, B83, B84, B85 Coxsackievirus B1, B2, B3, B4, B5, B6, A9
Enterovirus C	Coxsackievirus A1, A11, A13, A14, A19, A20, A21, A22, A24, Enterovirus C95, C96, C99, C102, C104, C105, C109, C113, C116, C117, C118. Poliovirus 1, 2 and 3.
Enterovirus D	Enterovirus D68, D70, D94, D111
Rhinovirus A	Human Rhinovirus A2, A7, A10, A59, A60, A61, A62, A63, A64, A11, A12, A13, A15 etc. (80 Serotypes)
Rhinovirus B	Human Rhinovirus B3, B4, B5, B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72 (32 Serotypes)
Rhinovirus C	Human Rhinovirus C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15 etc. (55 Serotypes)

1.4. MOLECULAR BIOLOGY

The infectious particle (virion) is about 30nm in diameter and is composed of a capsid protein which surrounds and protects its RNA genome. The capsid has an icosahedral symmetry and consist of four capsid proteins namely VP1, VP2, VP3 and VP4 each of which is repeated sixty times to form the virion (Shih *et al.*, 2004). Figures 1.4a and 1.4b are schematic diagrams of enteroviruses showing the various

capsid proteins and the RNA of the virus. The RNA molecule is about 7.5kb long and it is characterised by a VPg protein at the 5' end, an open reading frame and a poly-adenylated tail at the 3' end. All four capsids have similar structures (an 8-stranded anti-parallel beta barrel). VP1, VP2, VP3 and VP4 (entirely on the interior as shown in 1.4a) bind together to form a protomer. The protomer is repeated five times to form a pentamer and the pentamer is repeated twelve times to form the icosahedral virion (Hogle *et al.*, 1985).

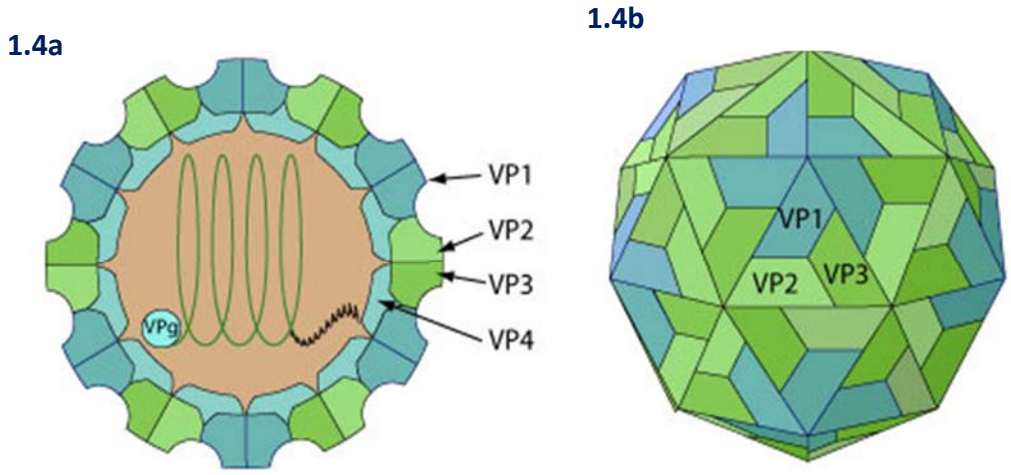


Figure 1.4a and 1.4b; schematic structures of enterovirus virion. 1.4a shows a disected view with the RNA genome and the capsid proteins whilst 1.4b shows the icosahedral structure. VP4 as shown in the diagram is exclusively on the interior (Diagram from Swiss Institute of Bioinformatics, not.dated)

When the RNA genome of the enterovirus is ultimately released into a susceptible and permissive cell upon infection, it is translated into the precursor proteins P1, P2 and P3 (polyproteins). These polyproteins are further processed to form the individual capsid and non-structural proteins. This is illustrated in a schematic diagram of the poliovirus genome is shown in figure 1.4c

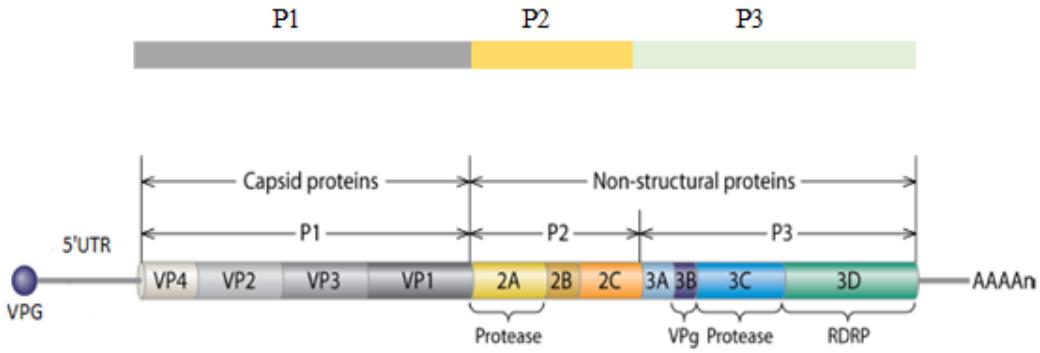


Figure 1.4c: Schematic representation of poliovirus genome from the polyproteins to the individual capsid and non-structural proteins (Jacobs *et al*., 2013). VP4, VP2, VP3 and VP1 constitute the capsid (structural) proteins encoded by the P1 precursor. The non-structural proteins include proteinases 2A and 3C and the 3Dpol which is an RNA-dependent RNA polymerase.

Replication cycle is first initiated by binding of the virion to its receptor, followed by internalisation into the infected cell and uncoating of the capsid protein to release the genomic RNA. The genome is translated into the polyprotein which is eventually cleaved to form the individual capsid and replication (non-structural) proteins (van der Linden *et al.*, 2015) as shown in Figure 1.4d. The non-structural proteins encoded by the virus are responsible for RNA replication through several protein-protein interactions between viral and/or host proteins to mediate RNA synthesis, induce the membranous vesicles and deliver the replication complex to the template (Sean and Semler, 2008). RNA replication occurs in the membranous vesicles that are induced upon infection.

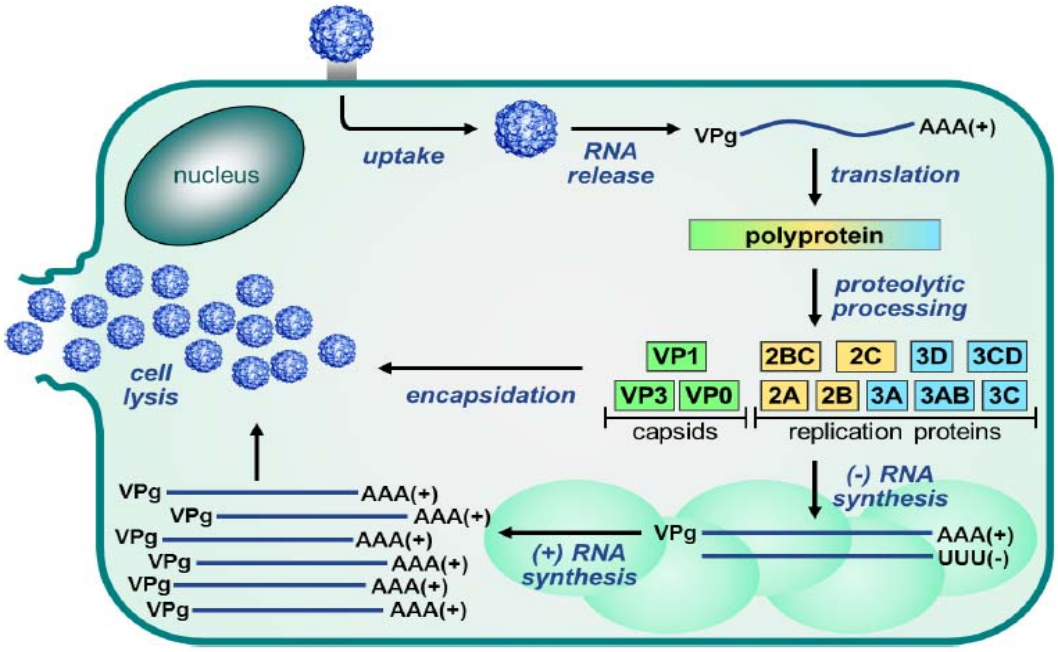


Figure 1.4d: Schematic diagram showing attachment and entry of viable virion into the cell, the replication process and the eventual cell lysis to release of new virions (van der Linden *et al.*, 2015)

Replication is initiated by uridylylation of the VPg (3B) protein by the 3DPol using the cis-acting RNA element (CRE) which is a stem loop structure as a template. The uridylylated VPg serves as a 'primer' binding to the poly-adenylated tail. It is then elongated by the 3Dpol to produce a negative RNA strand which then serves as an intermediate for the synthesis of more positive RNA strands. These positive RNA strands may either enter another round of translation or be packaged into capsids to produce virions (van der Linden *et al.*, 2015). Replication occurs outside the nucleus and the virions are released from the cell by cell lysis into the gastrointestinal tract. Virions are eventually excreted mostly by faecal egestion.

1.5. RECOMBINATION AND MOLECULAR TYPING

One of the major characteristics of enteroviruses is their ability to undergo recombination. The widely recognised model for recombination in enteroviruses is the 'copy choice' mechanism. According to this model, the RNA-dependent RNA polymerase (RdRp: 3Dpol in terms of enteroviruses) switches from one RNA molecule to another RNA molecule while remaining bound to the nascent nucleic acid chain, consequently generating an RNA molecule with mixed ancestry during replication. The presence of secondary structures in the RNA, the kinetics of transcription and extent of local sequence identity between the RNA templates are some of the factors that influence template switching and recombination (Simon-Loriere and Holmes, 2012). Recombination contributes to the high level of genetic variations observed in enteroviruses and can affect their evolution as well as viral pathogenesis (Lowry *et al*., 2014). It has been reported that recombination occurs mostly in the region of the *Enterovirus* genome that encodes the non-structural proteins. Some recombination have been reported in the 5' non coding region and VP4 region but at a lesser frequency compared to the non-structural genes. There are very few reports of recombination in the genes that encode the structural proteins VP1, VP2 and VP3. In a study assessing recombination in circulating enteroviruses at the 3D, VP1, 2A and 5'UTR genomic regions, strains of the same serotype clustered together in a phylogenetic tree only when the sequences in the VP1 genomic region was used. These suggests that the 5'UTR, 3D and 2A are more likely to undergo recombination and are hence not suitable for molecular typing of Enteroviruses (Lukashev *et al.*, 2003)

Initially, human enteroviruses were antigenically identified by antibody neutralisation tests (serology). Despite this process being generally reliable, it is labour intensive and in some cases may fail to identify the serotypes of a clinical isolate due to mitigating factors like antigenic drift, virus mixtures in specimens being tested and genetic recombination in the virus (Oberste et al., 1999a). It was suggested that the sequence of the capsid region VP1 should correspond with phylogenetic lineage since it contains a number of neutralisation domains (Oberste *et al*., 1999b) and is less likely to undergo recombination. Currently, human enterovirus types are identified by full or partial sequence on the VP1 or by seroneutralisation (Caro *et al*., 2001).

Identification with sequences from different regions other than the VP1 region can be useful as well. Targeting sequences of VP2 and VP4 capsid regions as an alternative method to type enteroviruses have been described (Nasri *et al*., 2007b). A study by Perera *et al* suggested that the nucleotide sequence encoding the VP2 protein could also be used for molecular typing of enteroviruses (Perera *et al*., 2010). The genome that codes for the VP4 region is however not reliable for typing.

1.6. DISEASE MANIFESTATIONS AND COST

Enteroviruses are genetically diverse with over 300 serotypes, about many of which are known to infect human beings. More recently, outbreaks of infections from *Enterovirus D68* in several parts of the world have been associated with severe respiratory illnesses mostly in people with asthma (Midgley *et al*., 2015).

CHAPTER 1: INTRODUCTION

Absenteeism from work or reduction in productivity at work due to a symptomatic infection, hospitalisation, effects on social life, costs associated with eradication initiatives and costs associated with purchasing treatment medicines contribute to the economic ramifications of *Enterovirus* infections. Common cold for example is caused mostly by viral respiratory tract infections and is the most common illness in human beings (Frederick *et al.,* 2003). Human rhinovirus species have been demonstrated to cause about 50% to 67% of all common cold symptoms (Mikaela *et al., 1998,* Arruda *et al*., 1997). Symptoms associated with common colds include rhinorrhoea, nasal congestion, sore throat, headache, malaise and cough. Common colds constitute a global health problem which affects activities like sleep, work performance, school and social life regardless of gender or age. In a study conducted in the United States in October 2001, the economic cost as a result of the common cold was estimated to be about $25 billion annually of which $16.6 billion was attributed to 'on the job productivity loss' (Bramley *et al*., 2002).

One of the most severe clinical manifestations that result from *Enterovirus* infection is poliomyelitis that could lead to partial or full paralysis. After the discovery of the polio vaccines, a number of countries have been declared polio free. Two main types of vaccine used are the IPV and OPV. IPV (Inactivated Polio Vaccine) are wild type poliovirus strains from the three serotypes which have been killed or inactivated with formalin. Also known as the 'Salk vaccine', it is administered by intramuscular injection. Since IPV is not live, it carries no risk of vaccine associated polio or any systematic adverse drug reaction. Although IPV is quite effective in conferring immunity, levels of intestinal immunity is quite low and as a result, immunised people infected with polio can still shed the virions through defaecation thereby

risking continued circulation. Shedding of poliovirus strains in faeces as a result low levels of immunity in the intestines means that it is possible to detect poliovirus in a sewage screen even if it has not been presented clinically for a considerable period. OPV on the other hand also known as 'Sabin vaccine' is a mixture of live attenuated or weakened poliovirus strains from the three serotypes and this confers immunity to the vaccinated person. Administered orally and relatively inexpensive compared to IPV, intestinal immune response to OPV is comparatively better and this means that mass vaccination can rapidly stop transmission of wild polio from an infected person to another. However, in some rare cases, OPV can cause paralysis

In countries where the disease remains endemic like Pakistan, Afghanistan and Syria, civil issues such as war have disrupted vaccine dissemination to the wider communities. Global Eradication Initiative projects that approximately $5.5 billion will be required to finance the eradication programme between 2013 and 2018 (Global Polio eradication initiative website, not dated).

South East Asia in particular have experience large outbreaks of *Enterovirus 71* infections since the late 1990s. *Enterovirus 71* which is an *Enterovirus A* serotype is one of the pathogens that cause hand foot and mouth disease (HFMD). This pathogen has been responsible for large outbreaks of HFMD outbreaks in Taiwan, Malaysia, China, Vietnam and Cambodia since the late 1990s (Sabanathan *et al*., 2014). In 1998, a large outbreak of *Enterovirus 71* in Taiwan resulted in 78 deaths. Subsequent smaller outbreaks recurred in 2000 and 2001. The outbreaks was recognised due to the large number of HFMD cases and the rapid deaths of young people who were affected (Lin *et al.*, 2008). Also, in Cambodia, 58 deaths were recorded in children as a result of *Enterovirus 71* outbreak between April and July 2012. The symptoms

were initial encephalitic presentation followed by destructive alveolar pneumonia which was rapid and often fatal (Sabanathan *et al.*, 2014). *Enterovirus 71,* was first isolated in 1969 in the state of California (Schmidt *et al.,* 1974) and since then, they have been isolated in many parts of the world. Large and small outbreaks of this pathogen since the late 1990s demonstrates the emergence and persistence of *Enterovirus 71* related diseases especially in South East Asia. There are no available vaccines against *Enterovirus 71* at the moment. Guidelines on how to manage hand foot and mouth disease has however been published by the World Health Organisation (WHO press, 2011). *Enterovirus 71* has also been associated with diseases like aseptic meningitis, encephalitis and cardiopulmonary dysfunction (Perez-Velez *et al.,* 2007)

1.7. ***THESIS***

As there are so many *Enterovirus* serotypes that cause a wide range of clinical syndromes, monitoring trends of *Enterovirus* infections in an area is important in the attempt to understand their temporal and geographical pattern. If people exhibiting clinical symptoms visit a medical facility and undergo medical diagnostic tests, data representing the serotypes circulating in an area can be obtained as part of *Enterovirus* surveillance. The major limitation to this form of surveillance is the fact that many *Enterovirus* infections are asymptomatic and hence such data will not be comprehensive. Taking poliovirus infection for example, only about one in hundred individuals infected with the virus will go on to exhibit paralytic symptoms.

Therefore, if there is no paralytic polio presentation clinically for some time, it does not necessarily mean that the virus is not circulating in the community.

The main mode of transmission of enteroviruses is the oral-faecal route and most infected people shed large amounts of virus in faeces for several days or weeks, both before and after onset of symptoms. Due to their stable capsid structure and conformation as well as their ability to withstand adverse conditions, virions of enteroviruses excreted into sewage can remain infectious for a considerable length of time. Circulation of enteroviruses in local sewage is therefore a marker of their presence in a particular community (Zheng *et al.*, 2013). Screening of sewage in a local area for the presence of enteroviruses would yield a more comprehensive data which will include strains that have not been presented clinically or asymptomatic. An example was the detection of wild type 1 poliovirus in a routine sewage screen from samples collected between the 7th and the 13th April 2013 in Southern Israel. Analysis of the strain indicated that it was introduced into that area in early February 2013 despite the fact that Israel had been declared Polio free since 2002 by the World Health Organisation (WHO). National supplementary immunisation with oral polio vaccine was consequently started in August 2013 (Anis *et al*., 2013).

Currently, there is limited data on circulating enteroviruses in the Edinburgh and no sewage surveillance to ascertain the types of enterovirus in the local area is being performed. With Seafield Treatment Waste Water Works serving as the sewage treatment centre for the city and its surroundings, a screen of sewage collected from there could equip us with the necessary information about the types of enteroviruses circulating in the city. The aim of this thesis was therefore to identify the *Enterovirus*

strains circulating in Edinburgh at different time points, assess what the temporal pattern and find out if they are clinically significant.

Enteroviruses were typed with the VP4 and partial VP2 genomic sequences after RNA extraction, RT- PCR, cloning and colony sequencing. Sequences were edited and aligned with the software SSE 1.2. Serotype identification was through the online algorithm BLAST and phylogenetic analysis (with MEGA6 software) to generate the relevant data for this thesis.

CHAPTER 2- MATERIALS AND METHODS

Materials and methods applied in this project were in two main categories:

i. Processing of sewage samples collected from Seafield Treatment Waste Water Works facility (laboratory based)
ii. Construction of datasets of DNA sequences and with the online algorithm BLAST to identify, the software SSE 1.2 to edit and align the sequences and the programme MEGA6 to perform test of phylogeny (In Silico based)

2.1. MATERIALS

2.1.1. Kits Used

i. SuperScript® III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen; Catalogue # 12574-018)
ii. QiaAmp Viral RNA mini kits (Qiagen; Catalogue # 52904)
iii. QIAquick Gel Extraction Kit (Qiagen; Catalogue # 28704)
iv. pGEM®-T and pGEM®-T Easy Vector Systems (Promega; Catalogue # A1360)
v. BigDye® Terminator v3.1 Cycle Sequencing Kit (Catalogue number: 4337455)
vi. GoTaq® G2 DNA Polymerase (Promega, Catalogue # M7841)

2.1.2. Machines

i. PCR Machine (Bibby Scientific; Techne TC-4000)
ii. Falcon 6/300 bench centrifuge (Product code: 12730616)

iii. New Brunswick INNOVA 4300 Series Shaker Incubator

iv. NanoDrop 1000 Spectrophotometer (NanoDrop Technologies V 3.7)

v. Eppendorf Centrifuge, Minispin/Minispin plus Microcentrifuge (Sigma-Aldrich, Product # Z606235)

vi. Rotor Gene- Q qPCR machine (QIAGEN; System serial number R0910 117)

vii. Heraeus Fresco 17 Centrifuge (Thermo electron corporation Catalogue # 75002420)

viii. G:Box Transilluminator (Syngene)

ix. Safe Imager Transilluminator (Invitrogen model S37102)

2.1.3. Reagents and Enzymes

i. RNase free Water (Qiagen; Catalogue 129112)

ii. 10mM dNTP (Deoxyribonucleotide triphosphate)

iii. RNA storage solution (Ambion UK, AM7001)

iv. LB Broth and LB Agar (Roslin Institute Laboratory)

v. E. coli competent cells (DH10β, Roslin Institute Laboratory)

vi. Ampicillin (Life Technologies; Catalogue number: 11593-027)

vii. IPTG reagent (Invitrogen™ Catalogue number: 15529-019)

viii. X-Gal (Invitrogen™ Catalogue number: 15520-034)

ix. 1kbp DNA Marker (Promega, Catalogue # G5711)

x. SYBR® Safe DNA Gel Stain (Invitrogen™ Catalogue number: S33102)

xi. UltraPure™ Agarose (Life Technologies Catalogue 16-500-100)

xii. 10µM DNA Primers (Refer to table 2.1a)

xiii. 10µM Random Hexamers

2.1.4. Miscellaneous

i. 50ml Falcon tubes

ii. 20ml syringe (Terumo Hypodermic; Ref Nos SS+ 20S1)

iii. 0.45µm syringe filter (Millipore; Catalogue # SLHV033RS)

iv. Centriprep YM-50, 50 kDa NMWL (Millipore, Catalogue # 4310)

v. Amicon Ultra-0.5 mL Centrifugal Filters (Millipore, Catalogue # UFC500308)

vi. X250 strip 8X tube and Domed caps (Thermoscientific item #10249393

vii. Petri dishes

viii. Eppendorf tubes.

A list of all the primers used in this thesis, their targeting regions and corresponding sequences is displayed table 2.1a.

Table 2.1a: Primers used in the thesis and their corresponding sequences

TARGETTING REGIONS	PRIMER	SEQUENCES
Outer Sense (VP4)	458s	CCGGCCCCTGAATGYGGCTAA
Inner Sense (VP4)	547s	ACCRACTACTTTGGGTGTCCGTG
Antisense (VP4)	1087as	TCWGGHARYTTCCAMCACCANCC
Antisense (VP4)	1178as	TCNGGRAAYTTCCARTACCANCC
Outer Antisense (VP4)	1125as	ACATRTTYTSNCCAAANAYDCCCAT
5'UTR – HEV-FWD	370s	GGCTGCGYTGGCGGCCTRC
5'UTR – HEV-REV	563as	ACACCCAAAGTAGTYGGTYCCR
M13 Forward (pGEM-T)	M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13 Reverse (pGEM-T)	M13R	TCACACAGGAAACAGCTATGAC

Composition of materials used in the experiment

1. Agarose Gel
    i. 2% UltraPure™ Agarose (Life Technologies Catalogue 16-500-100) in 1 x TAE solution
    ii. 0.0001% SYBR® Safe DNA Gel Stain (Invitrogen™ Catalogue number: S33102)
2. Agar Plates
    i. 0.5mM IPTG,
    ii. 8µg/ml X-Gal
    iii. 100µg/ml of Ampicillin.
    iv. LB Agar

2.2. METHOD

The experimental design used for this thesis with the aim of obtaining at least 30 *Enterovirus* sequences from each time point every fortnight between March 2014 and September 2014 is shown in Figure 2.2a.

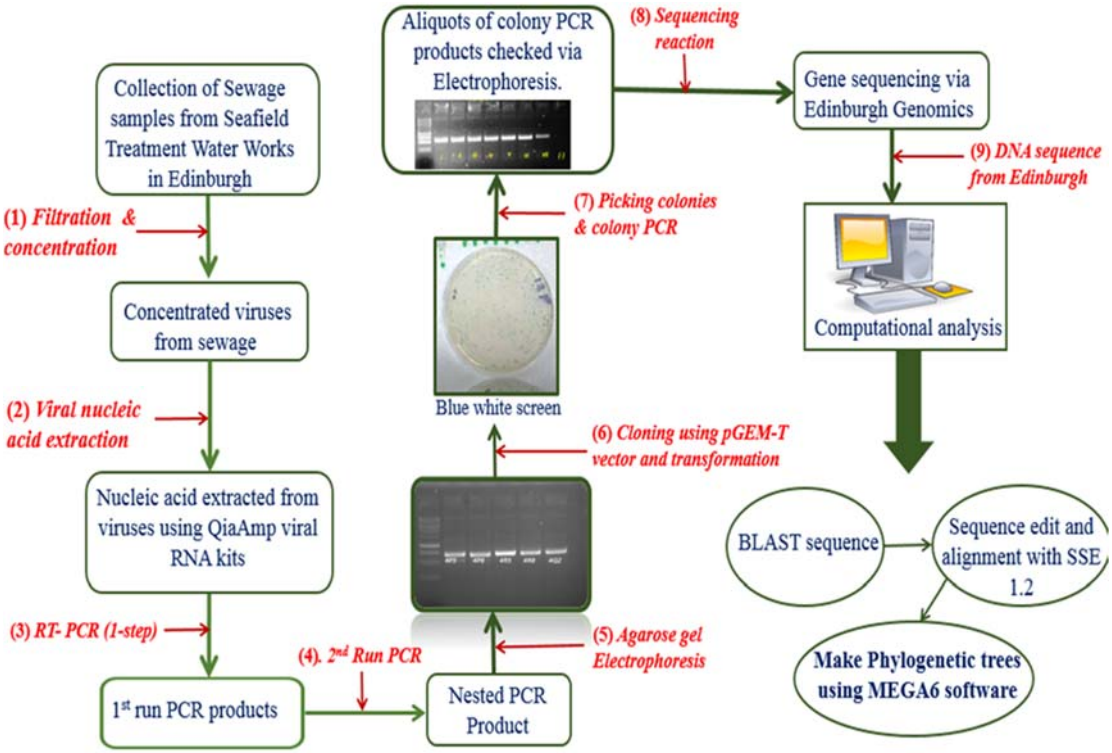


Figure 2.2a: Flow diagram showing the general experimental design for this thesis.

The Seafield Treatment Waste Water works Facility located at 20 Marine Esplanade, Edinburgh EH6 7RF was chosen as the site for collection of sewage samples because the facility treats waste water and sewage for a population of approximately 850000 people from Edinburgh which equates to about 300million litres of waste water daily from the city. It is hence a suitable source for monitoring enteroviruses circulating in

the greater Edinburgh area (waste water treatment and sewage, not dated). Pictures of the Seafield treatment water works facility are displayed in figure 2.2b and 2.2c.

Figures 2.2b and 2.2c: Seafield Treatment water works facility at 20 Marine Esplanade, Edinburgh EH6 7RF.

About 500ml of sewage sludge was collected and sent to the laboratory for further processing every fortnight. Table 2.2d refers to the time points of sewage sample collection and their respective dates.

Table 2.2d; Sewage collection time points and their corresponding dates

TIME POINT	DATE COLLECTED
1	26th March 2014
2	9th April 2014
3	23rd April 2014
4	7th May 2014
5	21st May 2014
6	4th June 2014
7	18th June 2014
8	2nd July 2014
9	16th July 2014
10	30th July 2014
11	13th August 2014
12	27th August 2014
13	10th September 2014

2.2.1. Control (detection limit of assay)

This was to determine the lowest concentration of viral RNA at which a PCR assay would remain sensitive. RNA serial (10 fold stepwise) dilutions of *Coxsackievirus A16* (*Enterovirus A), Echovirus 30 (Enterovirus B), Coxsackievirus 21 (Enterovirus C*) and *Enterovirus 70* (*Enterovirus D)* ranging from 10^6copies per µl to 10^{-1} copies per µl were prepared and three detection limit assays were performed on each of the solutions. To determine the detection limits, RT-PCR (SuperScript® III One-Step RT-PCR system with Platinum® Taq DNA Polymerase) and second round PCR was performed on the solutions and the products were visualised on a 2% agarose gel after electrophoresis to find the lowest RNA concentration at which the assay remained sensitive.

The one step RT-PCR reaction converts the viral RNA into cDNA and then amplifies the region of interest (VP4/Partial VP2 region) in a single reaction. Second run round PCR was performed after the one-step RT-PCR to improve sensitivity and specificity of the PCR process.

2.2.1a Preparation of RNA serial (10 fold stepwise) dilutions

Enterovirus stocks of *Coxsackievirus A16, Echovirus 30, Coxsackievirus A21 and Enterovirus 70* RNA representing *Enterovirus A-D* respectively were obtained from the Simmonds group and their respective concentrations were quantified using a NanoDrop ND-1000 with quantifying optical density at 260 nm. Assuming that the mean molecular mass of each nucleotide base is 330g/mol, the RNA concentrations were converted to RNA copy numbers. To prepare the 10 fold stepwise (serial) dilutions of RNA in copies per µl for each of the *Enterovirus* stocks, the RNA was

diluted in RNA storage solution (1mM sodium citrate, 0.1mM EDTA, pH 6.0: Ambion UK) containing 0.05µg/ml herring sperm carrier RNA and 0.1U/ml RNasin. Serial dilutions were either used in downstream reactions or stored at -80^{o}C.

2.2.1b Assays: amplifying VP4 and partial VP2 region

In assay 1, a single reaction mix for an RT-PCR contained 10µl of 2X Reaction mix, 0.7µl of primer 458s (10µM), 0.7µl of primer 1125as (10µM), 0.8µl of SSIII Taqman one-step and 3.8µl of nuclease free water. For the second run round PCR, a single reaction mix contained 12.7µl of Nuclease free water, 4µl of 5X Buffer Green Go Taq, 1µl of Primer 547s (10mM), 1µl of Primer 1087as (10mM), 0.2µl of 3mM dNTP, 0.1µl of G2 Go Taq and 1µl of RT- PCR product. The cycling conditions are shown in table 2.2.1b. In assays 2 and 3 however, the only procedural changes were the types of antisense primers used. Both assays 2 and 3 were hemi-nested PCR assays and the antisense primers used were 1087a and 1178a respectively (same antisense primer used in both RT-PCR and second round PCR). The reaction composition, sense primers and cycling conditions remained the same.

Agarose gel electrophoresis was performed using 2% agarose gel (1kbp DNA marker as a guide: expected size was 742 base pairs) and the image was visualised using the Safe Imager Transilluminator and a GBox gel imager to determine the detection limit of the assay.

Table 2.2.1b: Cycling conditions for RT-PCR and second run PCR for the detection limit assay

CYCLING CONDITIONS (ONE STEP RT-PCR)		CYCLING CONDITIONS (SECOND ROUND PCR)	
55°C for 30minutes	1 cycle	95°C for 15 seconds 52°C for 30 seconds 68°C for 50seconds	35 cycles
94°C for 2minutes			
95°C for 15 seconds	40 cycles		
52°C for 30 seconds			
68°C for 50seconds			
68°C for 5minutes	1 cycle		

2.2.2. Filtration and Concentration of Sewage/ RNA extraction

50ml falcon tube was filled with untreated sewage sludge collected from Seafield Treatment Waste Water Works and centrifuged in the Falcon 6/300 Sanyo MSE Centrifuge at 1100rpm for 15 minutes to sediment the sewage separating the supernatant from the solid particles. The supernatant was then drawn out with a 20ml syringe and filtered with a 0.45µm syringe filter (Millipore; Catalogue #SLHV033RS). The filtrate was then concentrated using a centriprep (YM-50 50 KDa NMWL: Millipore, Catalogue #4310) as described in the manufacturer's instructions. Further concentration to obtain the virus stock was done using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore) as described in the Ultra-0.5 mL Centrifugal Filters unit protocol

After obtaining the virus stock from filtration and concentration, RNA was extracted using QiaAmp Viral RNA Mini Kits as described in the kit manufacturer's instructions.

2.2.3. RT-PCR, and Agarose gel electrophoresis

Three RT-PCR and second round PCR assays were performed on the extracted RNA at each time point to amplify the VP4 and partial VP2 regions of *Enterovirus* genome using the same reagents, procedures and cycling conditions described in 2.2.1b. For all the assays, the DNA fragment sizes expected on the agarose gel after the PCR process and electrophoresis was 742 bases.

2.2.4. Cloning, colony PCR and sequencing

2.2.4a. DNA extraction: If DNA fragments (bands) had a size of approximately 742 bases (using the 1kb marker as a guide), the band was excised under UV light. DNA was then extracted from the excised bands with QIAquick Gel Extraction Kit according to the manufacturer's instruction to yield purified DNA.

2.2.4b. Ligation into pGEM-T-vector: Using the pGEM®-T and pGEM®-T Easy Vector Systems, generated purified DNA was ligated into the pGEM-T vector according to manufacturer's instructions

2.2.4c. Transformation into *E.coli* competent cells: 2µl of the ligation mix was pipetted into an eppendorf tube containing 50µl of thawed *E.coli* competent cells (DH10β) and this mixture was incubated on ice for 20minutes.

Then, 42^{o}C heat shock was applied to the mixture for 50 seconds after which the mixture was placed back on ice for 2minutes. 900µl of LB Broth was then added and the mixture was placed in a 37^{o}C shaker incubator for 75 minutes.

2.2.4d. Plating on the LB Agar/Amp/ IPTG/X-Gal Plates: 150µl of the transformation mix was streaked on an LB Agar/Amp/ IPTG/X-Gal plate and the plate was incubated overnight at 37^{o}C overnight. The pGEM-T vector has a gene that codes for ampicillin resistance and hence, the antibiotic ampicillin in the LB Agar/Amp/IPTG/X-Gal plate is to ensure that only *E.coli* successfully transfected with pGEM-T will be expressed.

2.2.4e. Blue white screen and colony selection: Several colonies grew on the LB Agar/Amp/IPTG/X-Gal plate and these colonies were either bluish or whitish in colour. The pGEM- T vector has a Lac Z gene which encodes the protein β-galactosidase. β-galactosidase hydrolyses X-gal (in the agar plate) by cleaving its β-glycosidic bond ultimately leading to the expression of 5'5 dibromo-4-4-dichloro indigo which is an intensely blue product. If the β-galactosidase is interrupted as a result of ligation, this hydrolysis of X-gal will not occur and therefore the blue coloured product is not expressed. Ligation interrupts the expression of the gene that encodes β-galactosidase meaning that blue colonies were not products of ligation. Only whitish colonies hence were selected for colony PCR and sequencing.

2.2.4f. Colony PCR: A single colony PCR reaction mix was composed of 13.7µl of nuclease free water, 4µl of Buffer Green Go Taq, 1µl of 10µM M13 Forward primer, 1µl of 10µM M13 Reverse primer, 0.2µl dNTP, 0.1µl of G2 Taq Polymerase and the selected colony (whitish colony harvested with a pipette tip and dipped into the reaction mix). In a PCR reaction, the M13 forward and reverse primers bind to the pUC/M13 forward and reverse sequencing primer binding sites respectively on the pGEM-T vector. These sites are adjacent to the DNA insert and hence, a successful PCR using the M13 primers will amplify both the M13 regions as well as the DNA insert. The expected size of the amplicon after colony PCR was 979 bases. The cycling conditions for colony PCR is shown in Table 2.2.4f.

Table 2.2.4f: Cycling conditions for colony PCR for the detection limit assay

CYCLING CONDITIONS (COLONY PCR)	
95ºC for 20 seconds 55ºC for 45 seconds 70ºC for 50seconds	35 cycles

2.2.4g. Sequencing reaction: For each product, two reactions were performed with different primers; one with the M13 forward primer and the other with the M13 reverse primer. Using the BigDye® Terminator v3.1 Cycle Sequencing Kit, the composition or the reaction mix and the cycling conditions of the PCR is shown in Table 2.2.4g

Table 2.2.4g: Reaction mix and the cycling conditions used in the sequencing reaction

SEQUENCING REACTION MIX	CYCLING CONDITIONS	
	95°C for 10min	Hold
8µl RNase free water 0.6µl Big Dye solution 1µl 10µM M13 primer 0.4µl Colony PCR product (979 bases)	96°C for 3 sec 62°C for 15sec 68°C for 30sec	35 cycles
	72°C for 2min	Hold

Products were shipped to Edinburgh Genomics (Edinburgh Genomics, Ashworth Laboratories, Charlotte Auerbach Road, The King's Buildings, The University of Edinburgh, EH9 3FL, Edinburgh, Scotland) where they were sequenced.

2.2.5 BIOINFORMATICS (IN –SILICO BASED)

Sequences of the colonies were downloaded from the Edinburgh Genomics website in text and chromat files. Sequences from the text file were loaded unto the SSE 1.2 programme where they were edited with critical visual perception using the chromat files as a guide. The editing included the removal of the 5'UTR sequences and the primer binding sites. After editing, a complete fragment of DNA was about 458 bases. Sequences were also loaded unto the BLAST (Basic Local Alignment Search Tool) algorithm to be establish the species. BLAST only identifies the sequences on its database that best resemble the query sequence. Species specific phylogenetic analysis was then performed to determine the serotypes.

Phylogenetic analysis are performed to study the evolutionary history of the sequences obtained and their relationship to the reference sequences from Genbank which is an open access sequence database produced and maintained by the National Centre for Biotechnology Information (NCBI). Using the software for constructing phylogenetic trees known as MEGA6 (Molecular Evolutionary Genetic Analysis), neighbour joining phylogenetic trees with 500 bootstrap replications using maximum composite likelihood and pairwise deletions were constructed to identify serotypes

2.2.5b. Typing with partial VP2 region - The VP1 region of *Enterovirus* genome has been shown to correlate with their serotypes (Oberste *et al*., 1999a). However, RT-PCR targeted the VP4 and partial VP2 regions in all the three assays. VP4 region of the *Enterovirus* genome is highly prone to recombination (Lukashev *et al.,* 2003) and therefore not reliable for molecular typing. Sequences of *Enterovirus species* detected in sewage were 458 nucleotide bases long (the first 203 for the VP4 region and 255 bases from the VP2 region). To test whether the first 255 nucleotide sequences of the VP2 region were sufficient for molecular typing, sequences of human enteroviruses were downloaded from Genbank and phylogenetic trees were constructed using the first 255bases of their VP2 region to assess whether sequences from the same serotype clustered together.

2.2.5b Identifying serotypes: After editing the sequences from the sewage, they were aligned with reference sequences for all the established human *Enterovirus* species using the SSE 1.2 software. The first 255 bases from the VP2 region were selected for phylogenetic analysis to identify the serotypes and generate the relevant data for this thesis.

CHAPTER 3: RESULTS

3.1. **LABORATORY BASED RESULTS**

The detection limit for all the three assays was determined to be 10^1 RNA copies per µl for *Enterovirus A-D.* Similar sensitivities across the four species meant that the rate of detection will not significantly lead to bias selection during PCR. Figure 3.1a is a diagram showing the agarose gel results from the detection limit assay for assay 1. All three assays showed similar results.

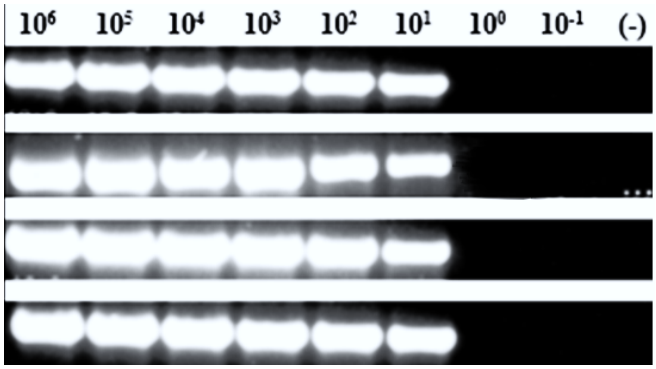


Figure 3.1a shows the detection limit assay visualised on an agarose gel. These were 10 fold stepwise dilutions of RNA (10^6 to 10^{-1} copies per µl)

All 13 pre-treatment sewage samples were RT-PCR positive for human enteroviruses in all the three assays. Figure 3.1b is the result of agarose gel electrophoresis after RT-PCR and second round PCR. Expected DNA size was 742 bases

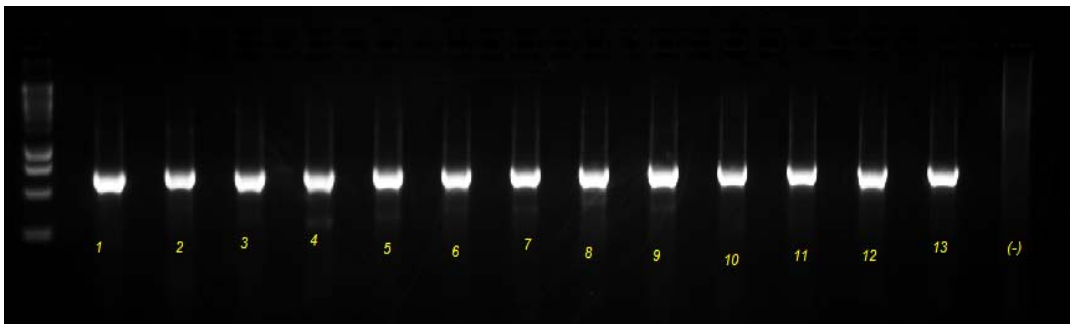


Figure 3.1b: Agarose gel photo for assay 3. All 13 time points were positive for human enteroviruses.

After the Blue white screen, colony PCR was performed. Figure 3.1c shows the result of the second time colony PCR for assay 3.

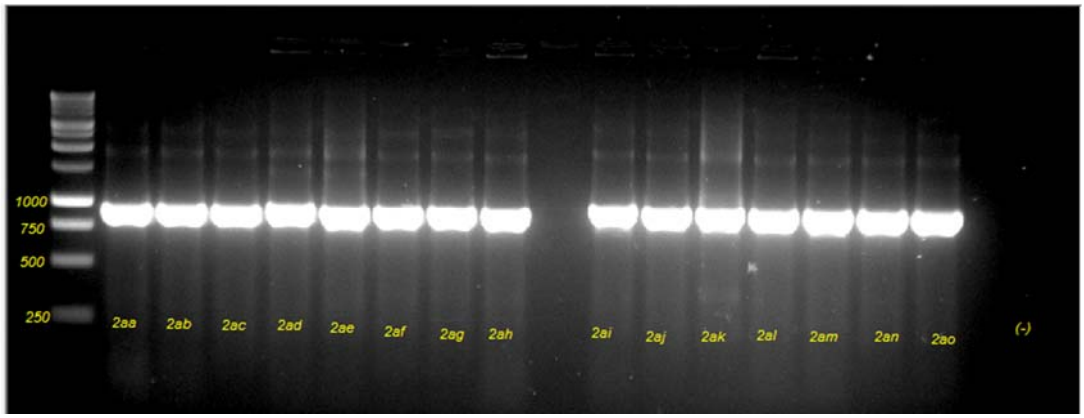


Figure 3.1c: result of a colony PCR on an agarose gel.

PCR products whose band size on an agarose gel were approximately 979 bases were selected for sequencing.

3.2. IN SILICO BASED RESULTS

After sequence BLAST, it became clear that none of the serotypes identified belonged to *Enterovirus D*. This was not a surprise as *Enterovirus D* species are largely respiratory tract infections. Apart from the conspicuous absence of *Enterovirus D,* serotypes from all the other *Enterovirus* species were identified in the sewage samples collected across the 6 months study period.

3.2.1a. Testing VP2 for molecular typing: Due to the high level of sequence diversity, designing primers that anneal specifically to the genome to amplify the VP1 genomic region for all the species of enteroviruses is quite challenging. Although RT-PCR to amplify the VP4 and partial VP2 regions were sensitive at 10^1 copies/µl of RNA for *Enterovirus A-D* in the detection limit assays, the VP4 region is more prone to recombination and is therefore not reliable for molecular typing. The VP4 and partial VP2 genomic regions targeted by the RT-PCR was about 458 nucleotide bases long (first 203 bases for VP4 and the next 255 bases for partial VP2) after editing. To test whether the first 255 nucleotide bases in the VP2 region was sufficient for molecular typing, reference sequences (refseq) of established human *Enterovirus* serotypes across species A, B and C were downloaded from Genbank and phylogenetic analysis were performed with the first 255 nucleotide bases of the VP2 genomic region to assess whether sequences from the same serotype clustered together (*Enterovirus D* was ignored as there were no serotypes identified in sewage). As shown in Figure 3.2.1.ai (*Enterovirus A*), 3.2.1aii (*Enterovirus B*) and 3.2.1aiii (*Enterovirus C*), sequences from the same serotype clustered together meaning that the first 255 nucleotide bases of VP2 region could be used for molecular typing.

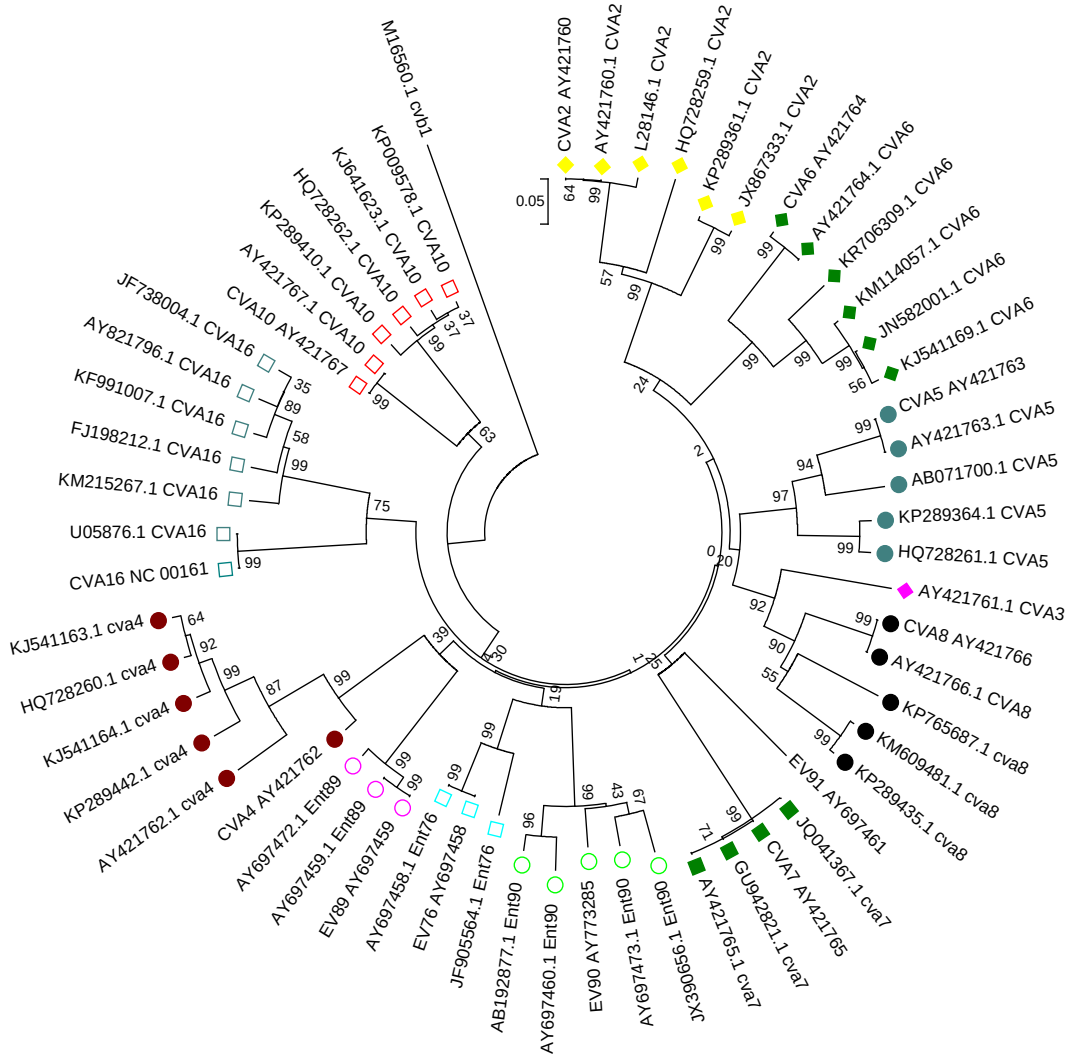


Figure 3.2.1ai: The first 255 nucleotide bases in the VP2 regions of *Enterovirus A* serotypes in a neighbour- joining tree. VP2 sequences from the same serotypes cluster together. This means that the partial VP2 region displays 100% concordance with their respective serotypes suggesting that the region is sufficient for molecular typing

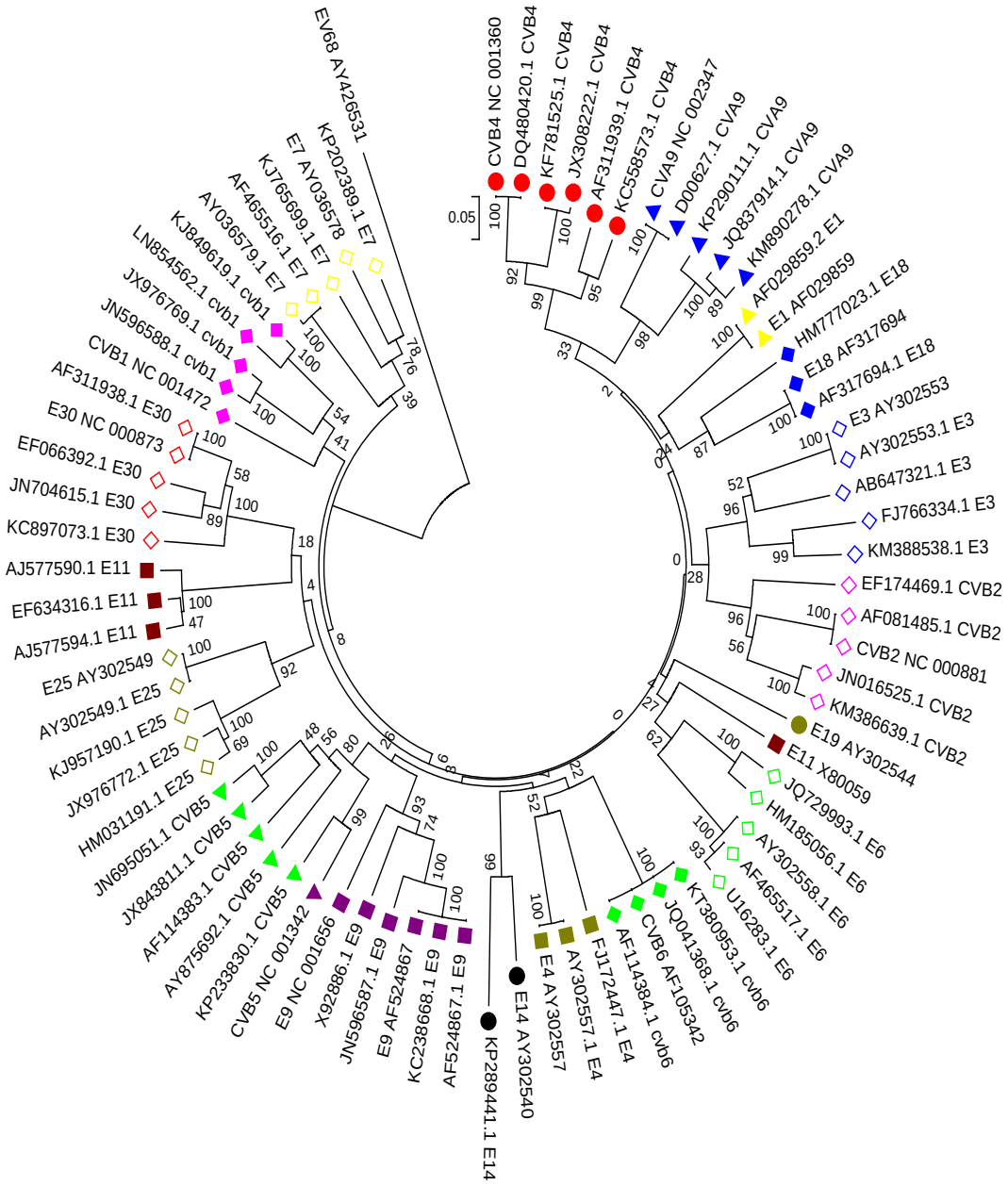


Figure 3.2.1aii: The first 255 nucleotide bases of the VP2 regions of *Enterovirus B* serotypes in a neighbour- joining tree. VP2 sequences from the same serotypes cluster together. This means that the partial VP2 region displays 100% concordance with their respective serotypes suggesting that the region is sufficient for molecular typing

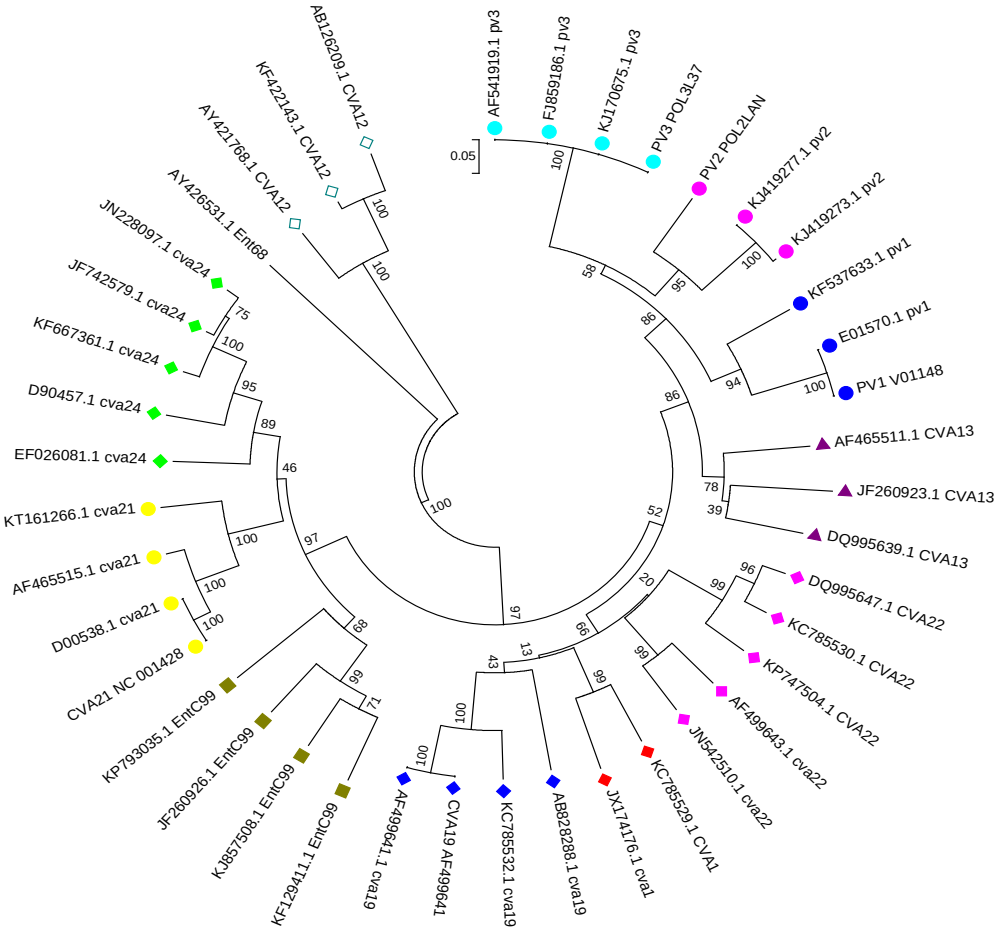
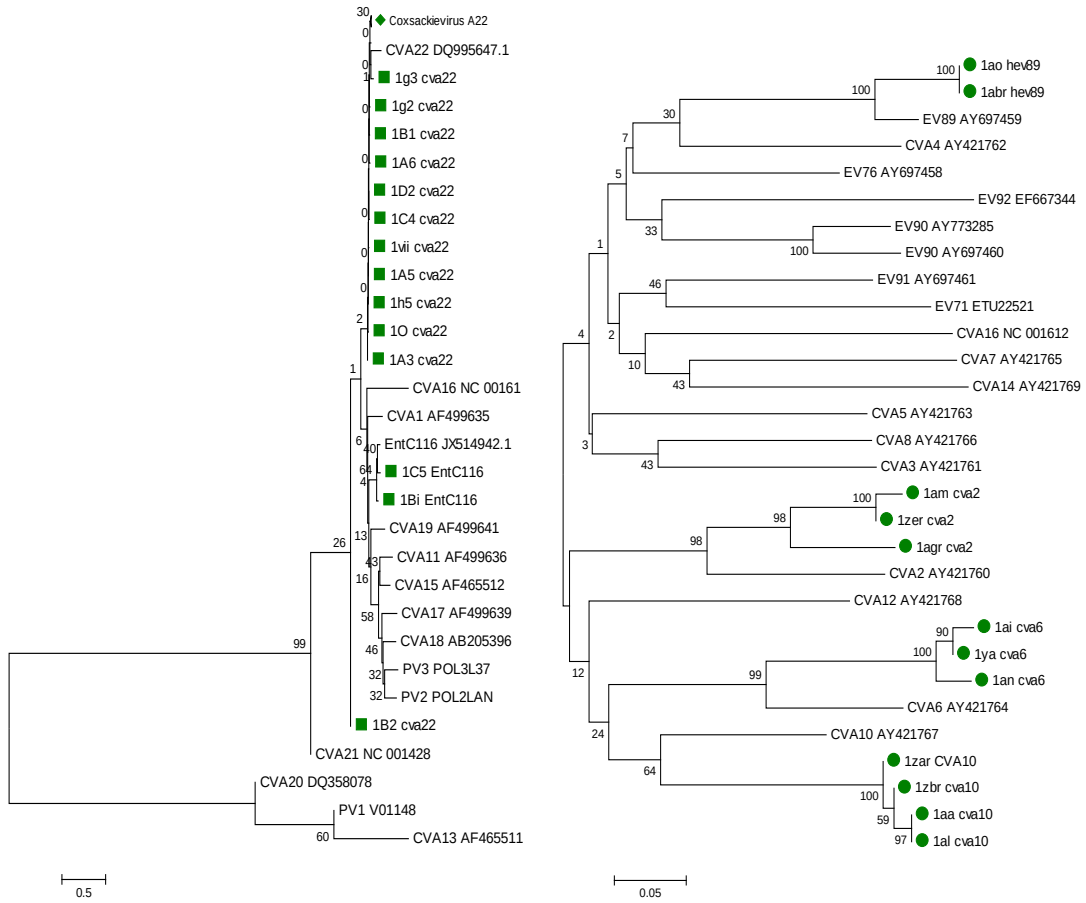


Figure 3.2.1aiii: The first 255 nucleotide bases of the VP2 regions of *Enterovirus C* serotypes in a neighbour- joining tree. VP2 sequences from the same serotypes cluster together. This means that the partial VP2 region displays 100% concordance with their respective serotypes suggesting that the region is sufficient for molecular typing

3.2.1b. Serotype identification and distribution: After sequences of colonies were obtained from Edinburgh Genomics and identified by BLAST, they were grouped into their respective species (Species A, B, C and Rhinovirus) for each time point. After alignments with reference sequences for all established human enteroviruses, the serotypes were identified by phylogenetic analysis. Figure 3.2a, 3.2b and 3.2c shows phylogenetic trees used to identify serotypes detected in sewage that was collected on the 26th of March 2014 (time point 1)



Figures 3.2a and **3.2b:** Phylogenetic trees (neighbour joining trees) to identify serotypes using 255 bases of the VP2 genome region. Sewage samples were collected on the 26th of March 2014. 3.2a is for the serotypes belonging to Enterovirus C whereas 3.2b are for serotypes belonging to Enterovirus A. Serotypes of samples are labelled in green.

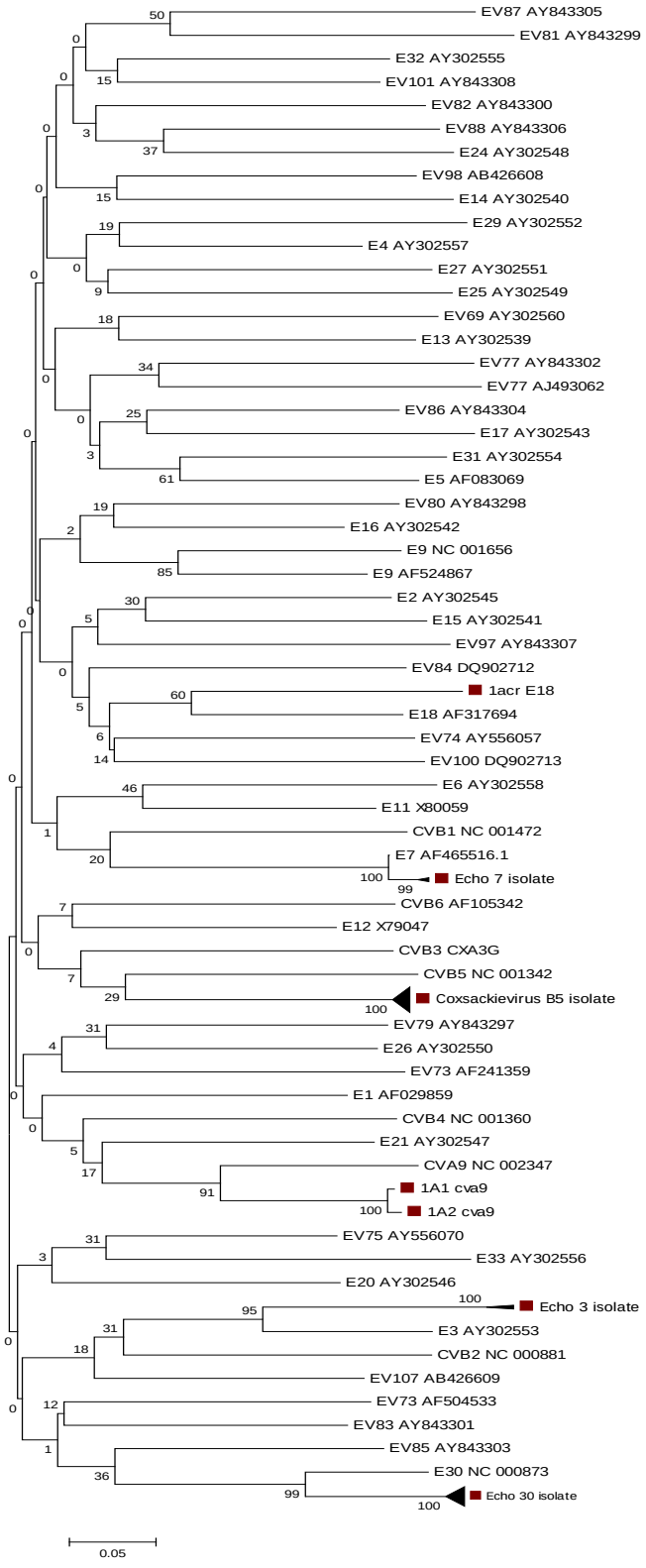


Figure 3.2c: Phylogenetic tree to identify the *Enterovirus B* serotypes detected in sewage collected on the 26th of March 2014. Serotypes from sewage labelled with red squares.

A total 598 sequences were obtained constituting 31 serotypes (7 belonging to *Enterovirus A*, 16 to *Enterovirus B* and 8 to *Enterovirus C*) across the three assays. Some Rhinoviruses were also identified.

Despite the fact that the detection limit was 10^1 RNA copies per µl, it was a bit surprising that no *Enterovirus A* serotypes were picked up in assay 1. Apart from that, there were a few instances (time point 8, time point 12 and time point 13) where only *Coxsackievirus A22* strains were detected in that same assay. A careful examination of the in house primer 1125a (outer antisense primer) showed that there was a mismatch in the first three bases of the primer at the 5' end with the primer binding site of *Enterovirus A* species with the exception of *Coxsackievirus A16* which is why the detection limit assay worked. There was therefore preferential binding to other *Enterovirus* species. Assays 2 and 3, both hemi-nested were performed and they were more sensitive to *Enterovirus A* serotypes in the same region.

Merging the three assays, at least 30 sequences were obtained for each time point. The serotypes identified and their percentage abundance at each time point is illustrated in Figures 3.2.1a to 3.2.1m.

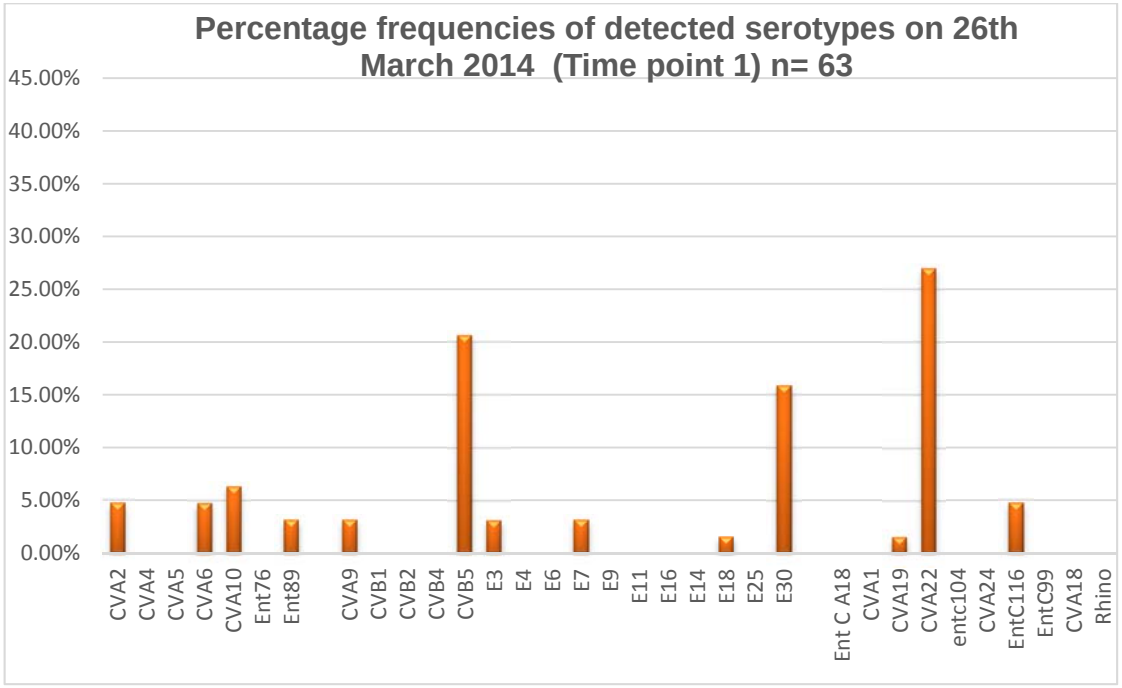


Figure 3.2.1a; Percentage frequencies of identified serotypes at time point 1

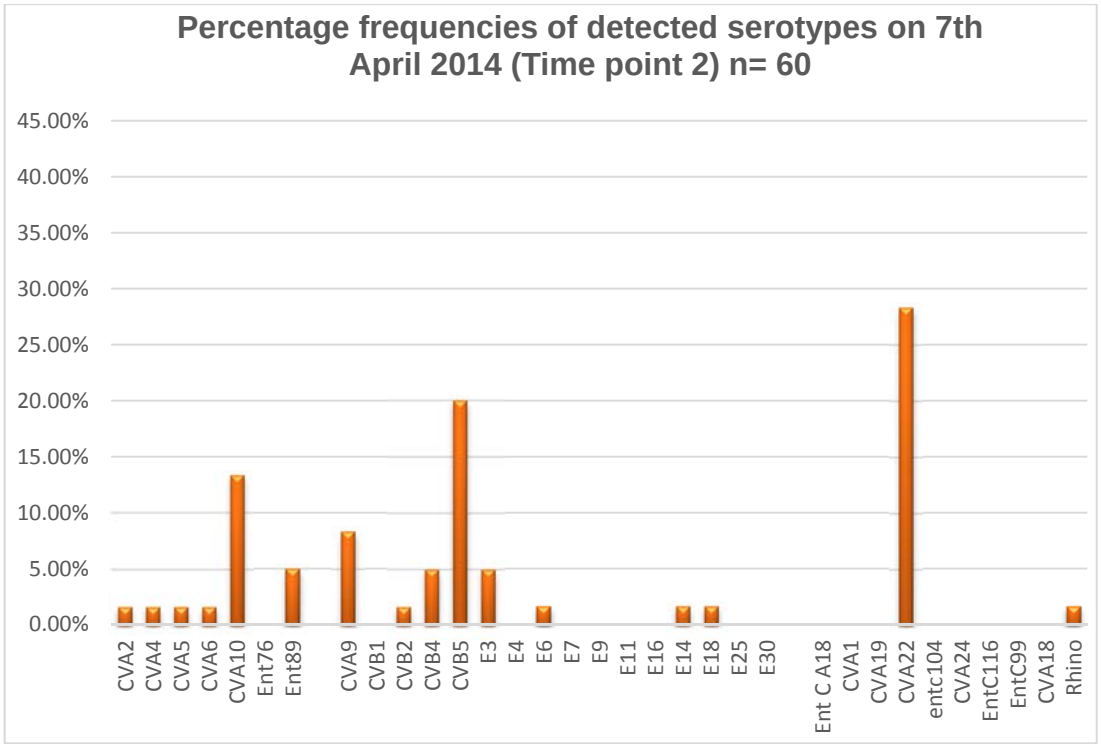


Figure 3.2.1b; Percentage frequencies of identified serotypes at time point 2

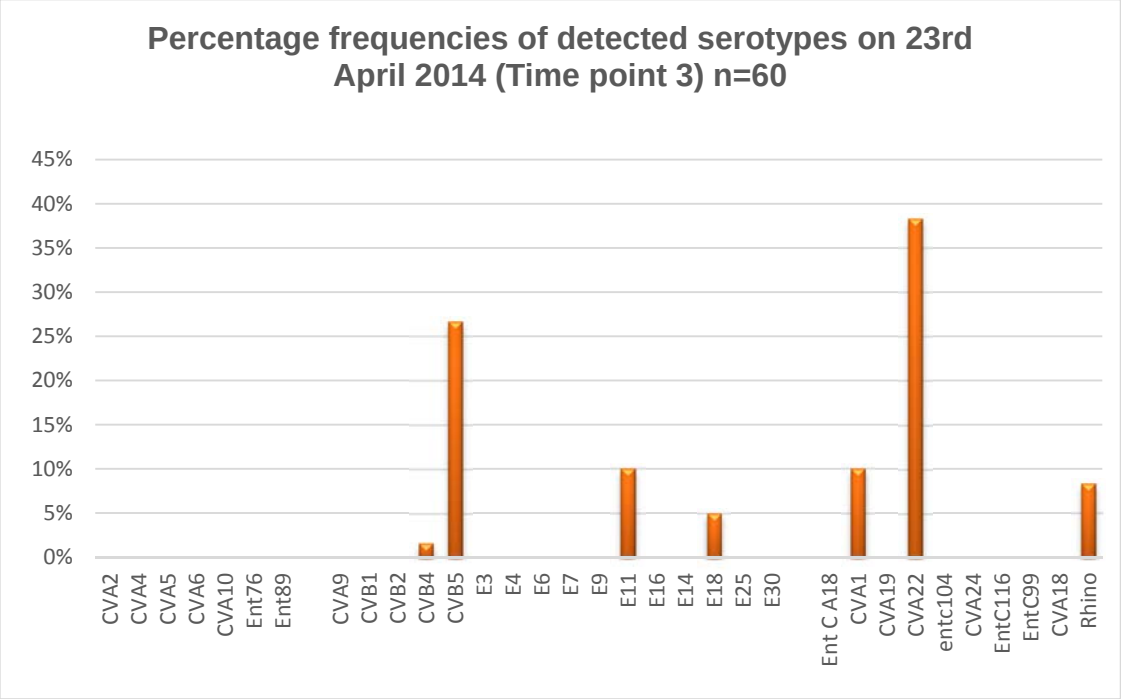


Figure 3.2.1c; Percentage frequencies of identified serotypes at time point 3

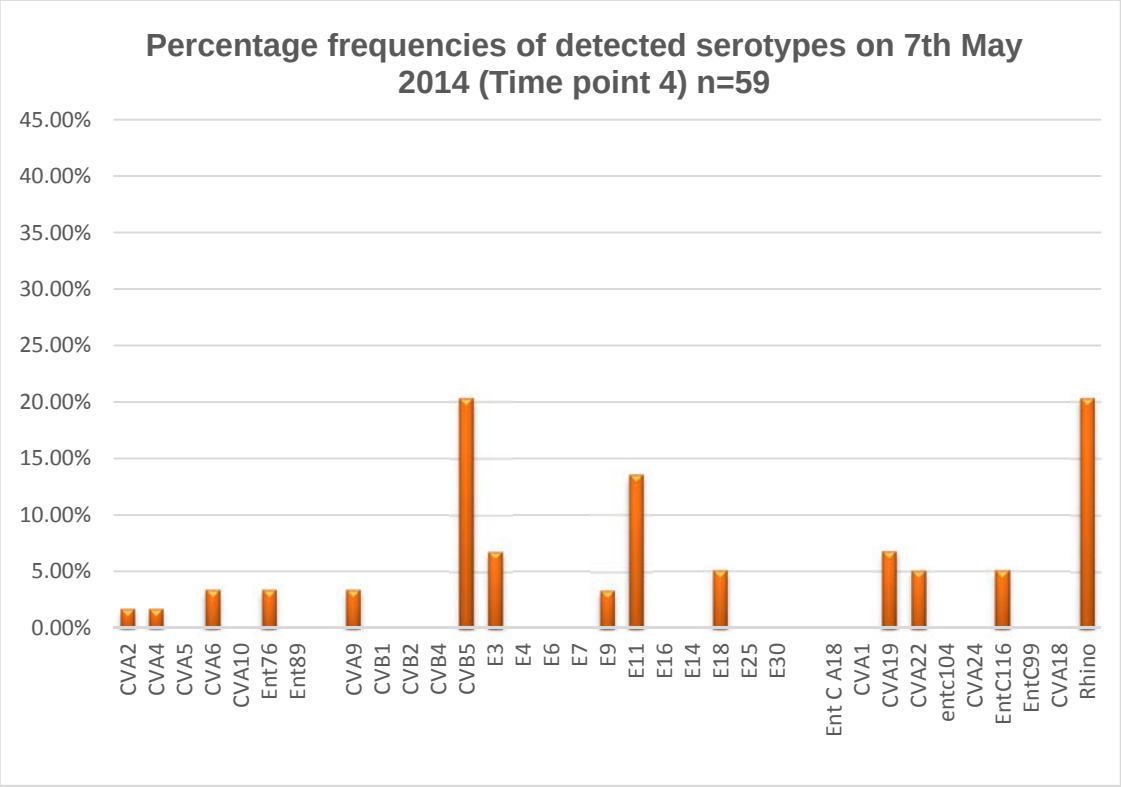


Figure 3.2.1d; Percentage frequencies of identified serotypes at time point 4

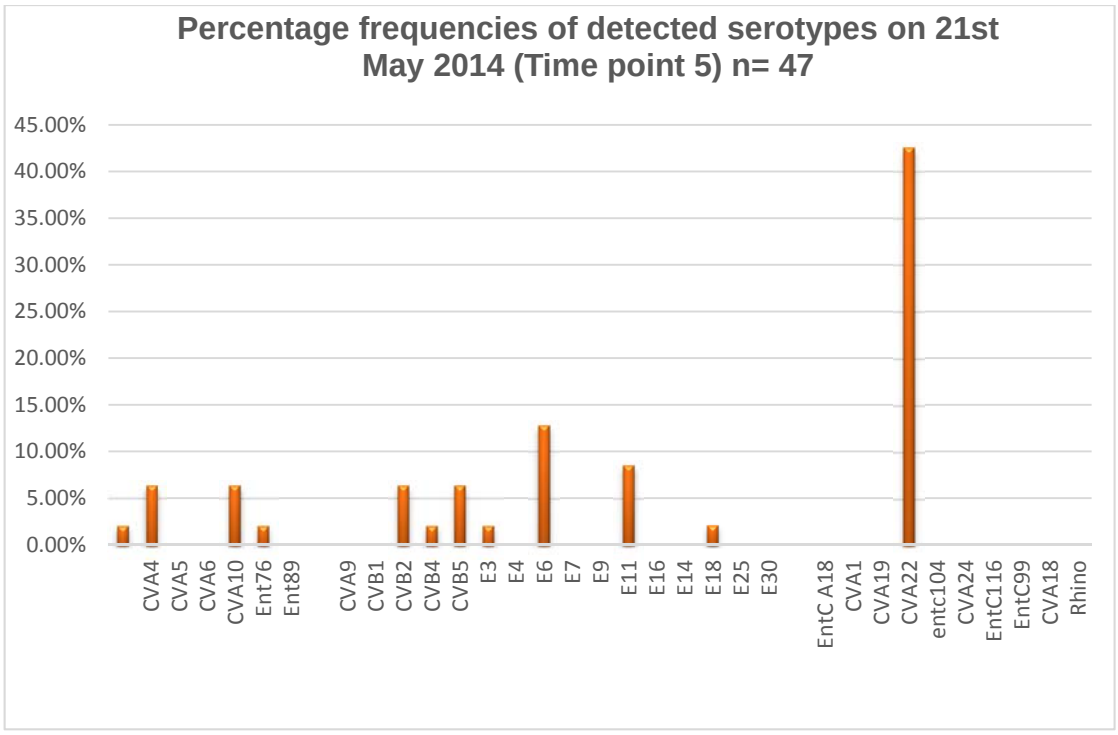


Figure 3.2.1e; Percentage frequencies of identified serotypes at time point 5

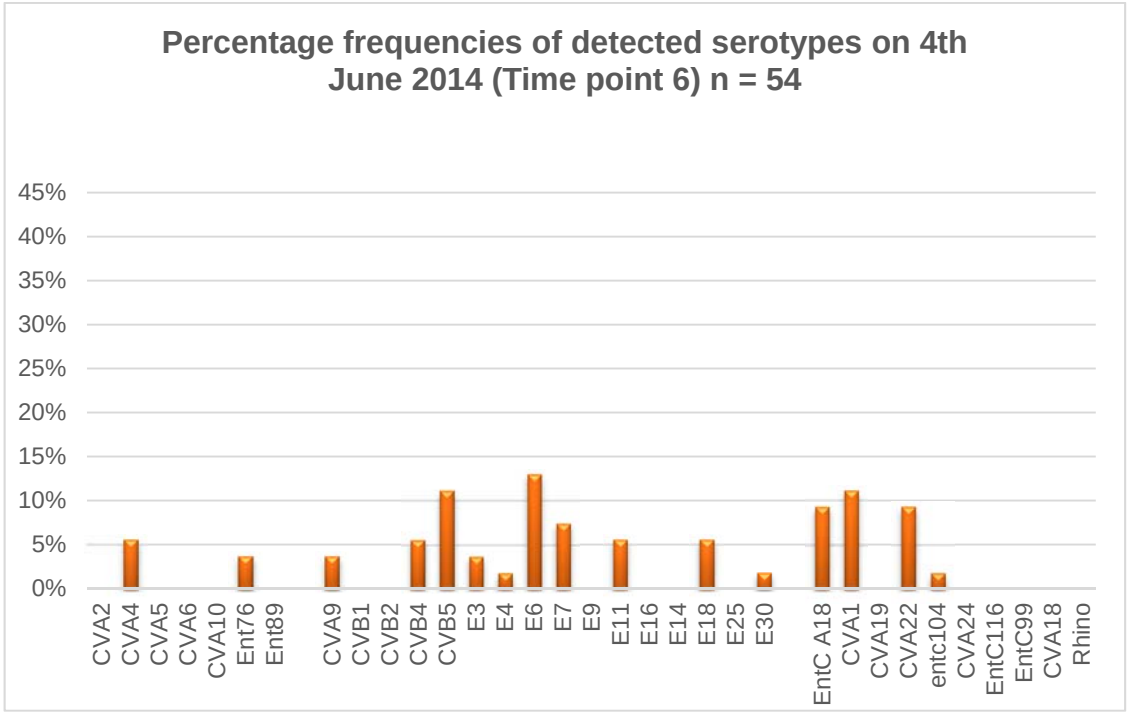


Figure 3.2.1f; Percentage frequencies of identified serotypes at time point 6

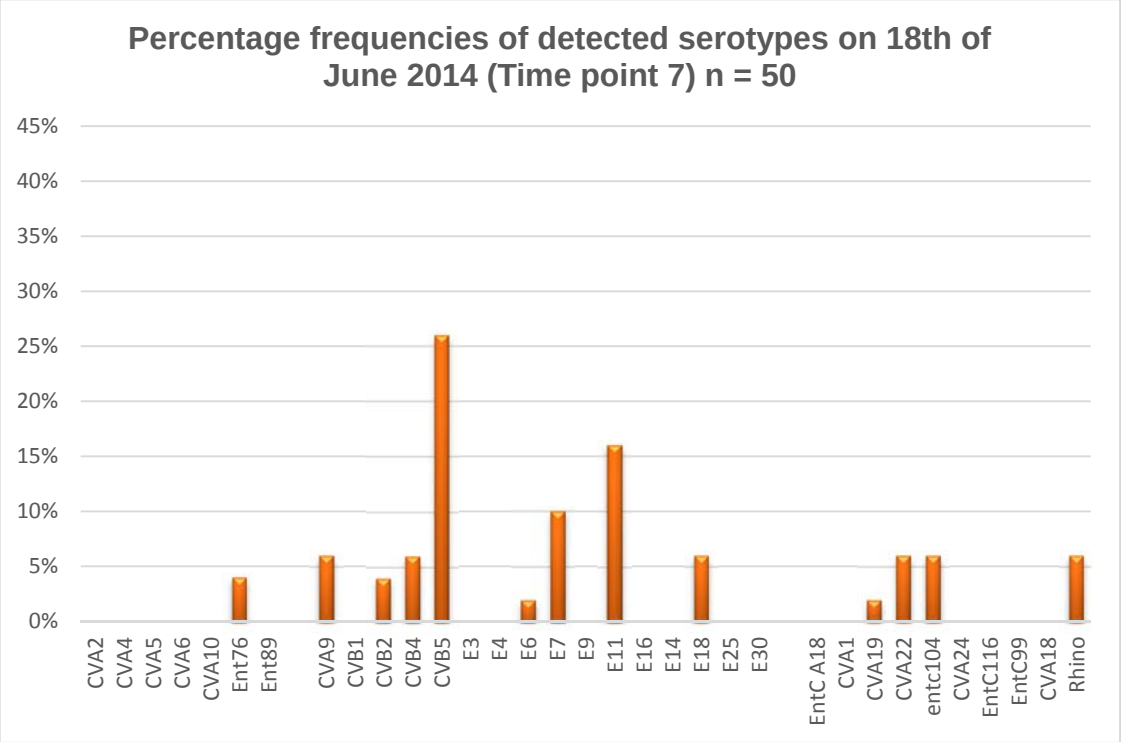


Figure 3.2.1g; Percentage frequencies of identified serotypes at time point 7

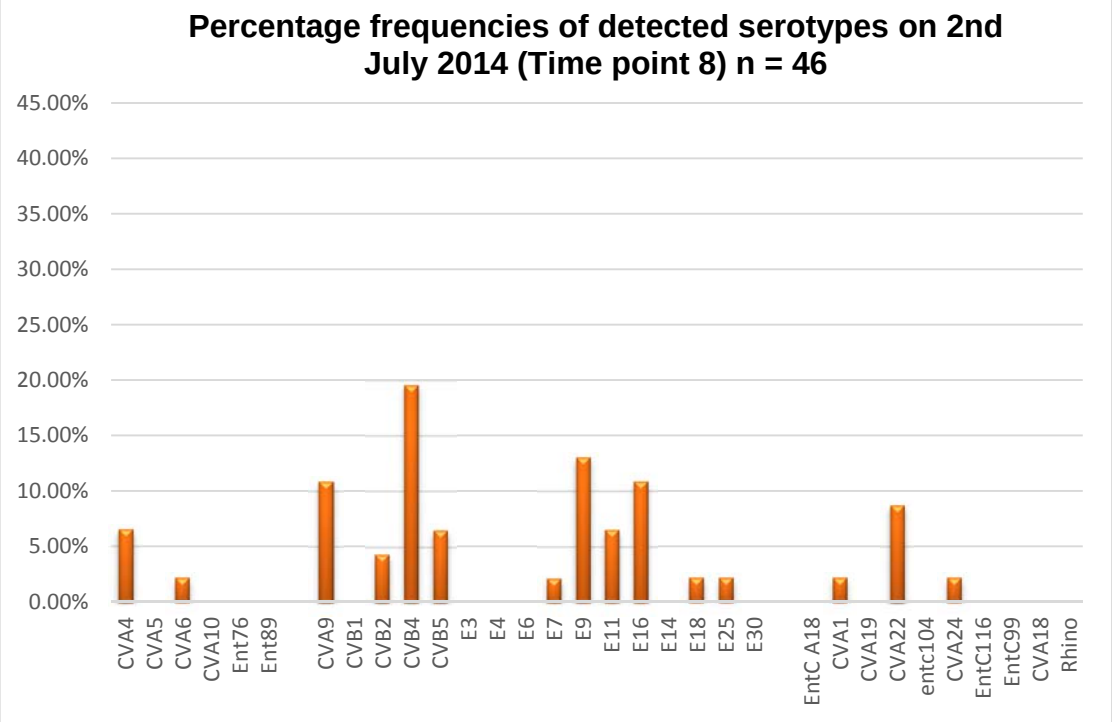


Figure 3.2.1h; Percentage frequencies of identified serotypes at time point 8.

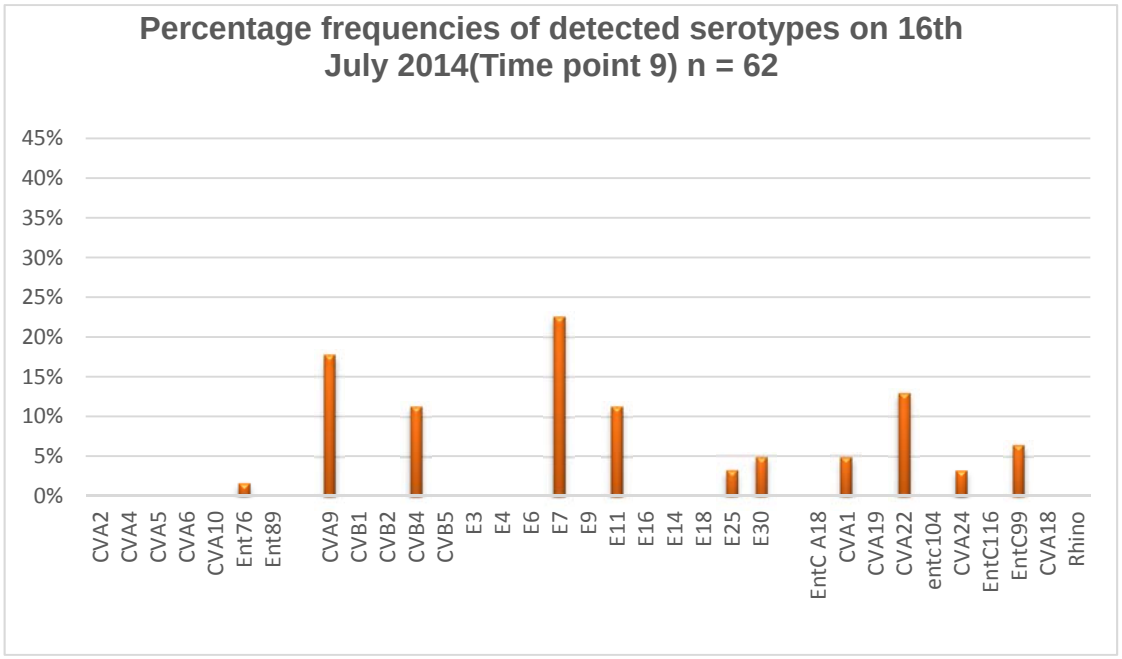


Figure 3.2.1i; Percentage frequencies of identified serotypes at time point 9

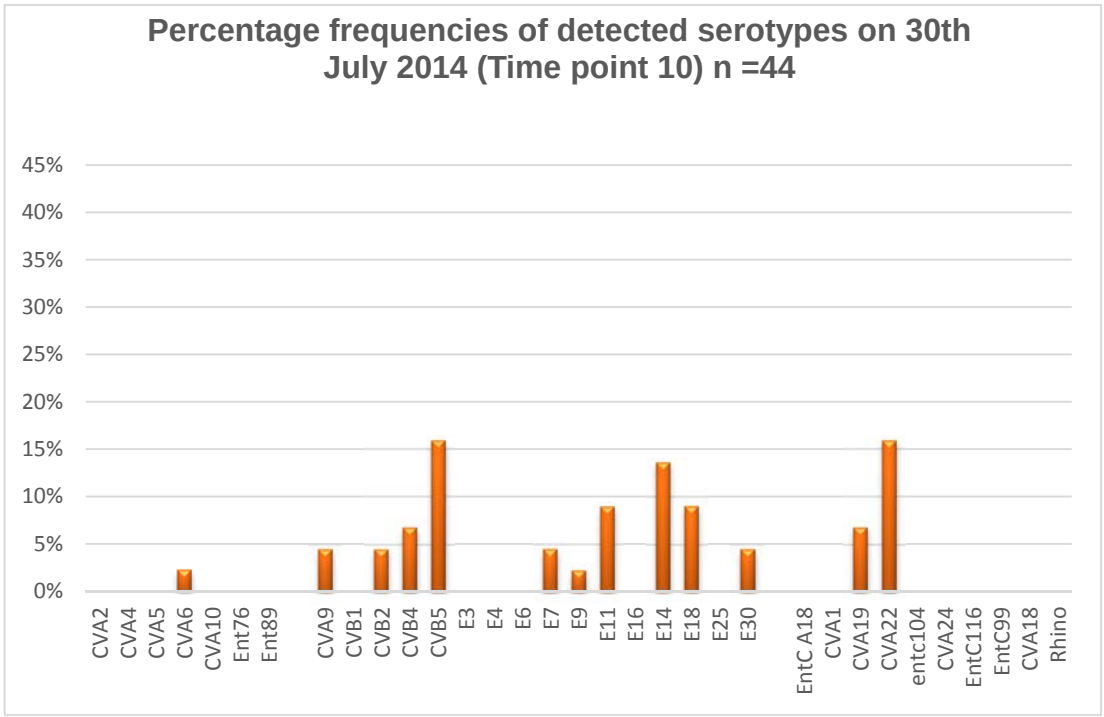


Figure 3.2.1j; Percentage frequencies of identified serotypes at time point 10

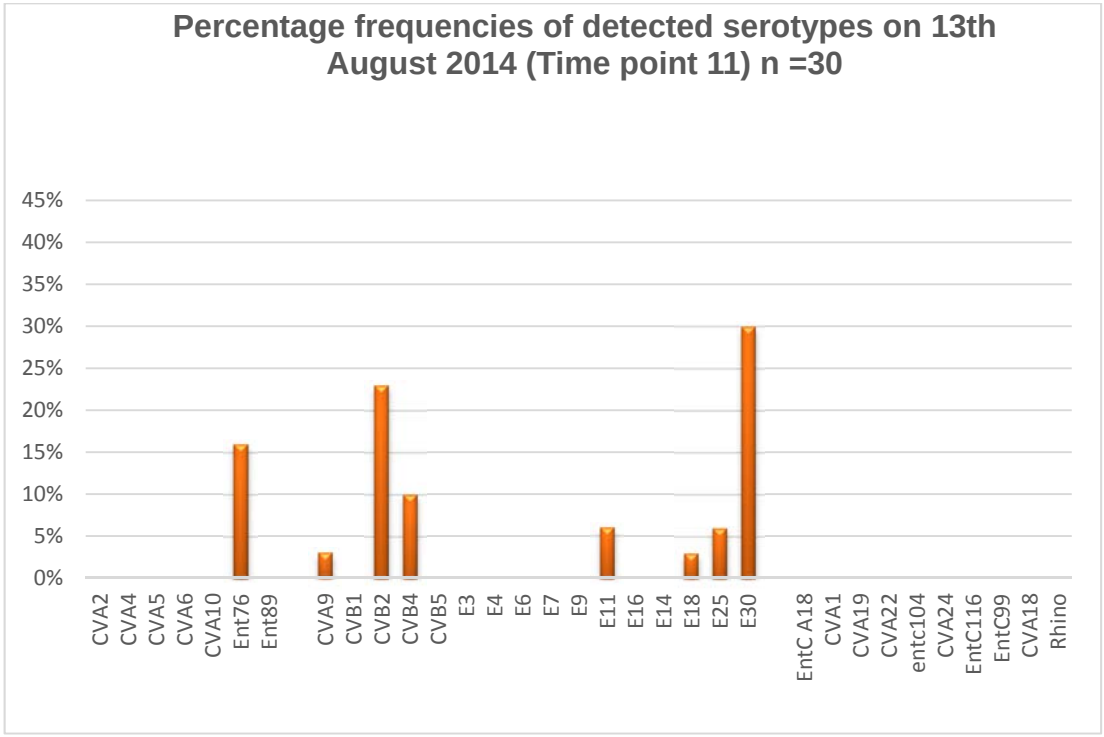


Figure 3.2.1k; Percentage frequencies of identified serotypes at time point 11

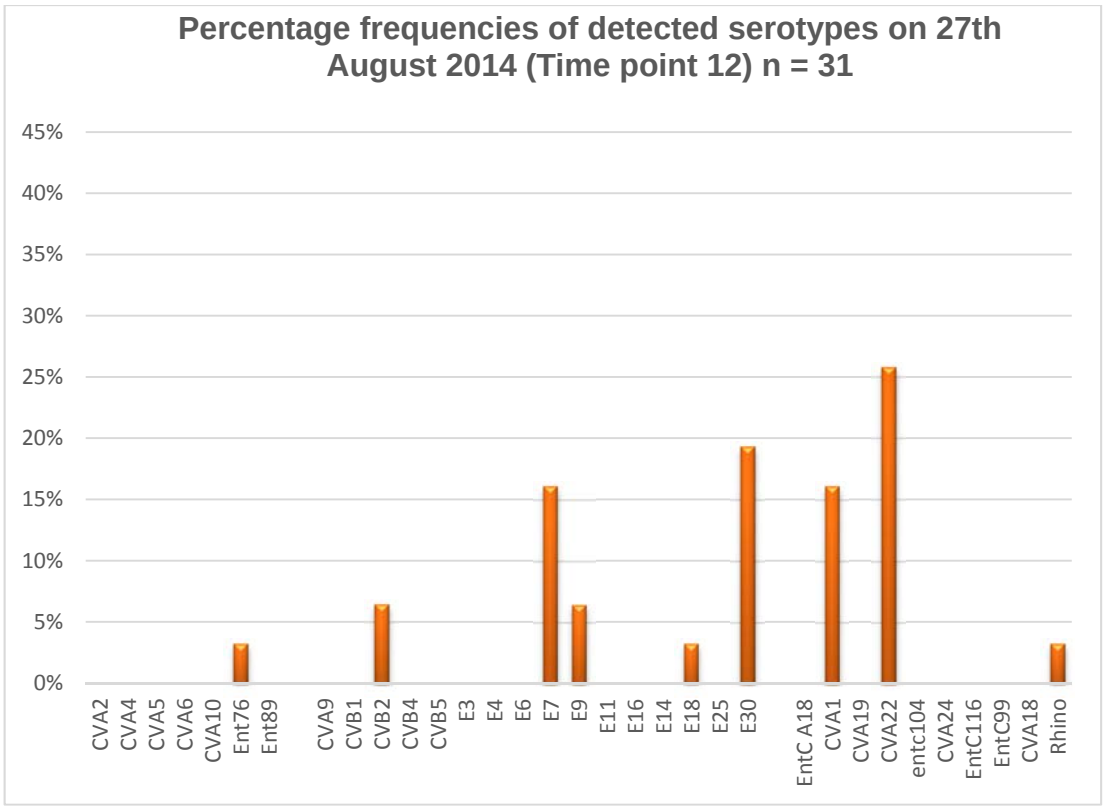


Figure 3.2.1l; Percentage frequencies of identified serotypes at time point 12

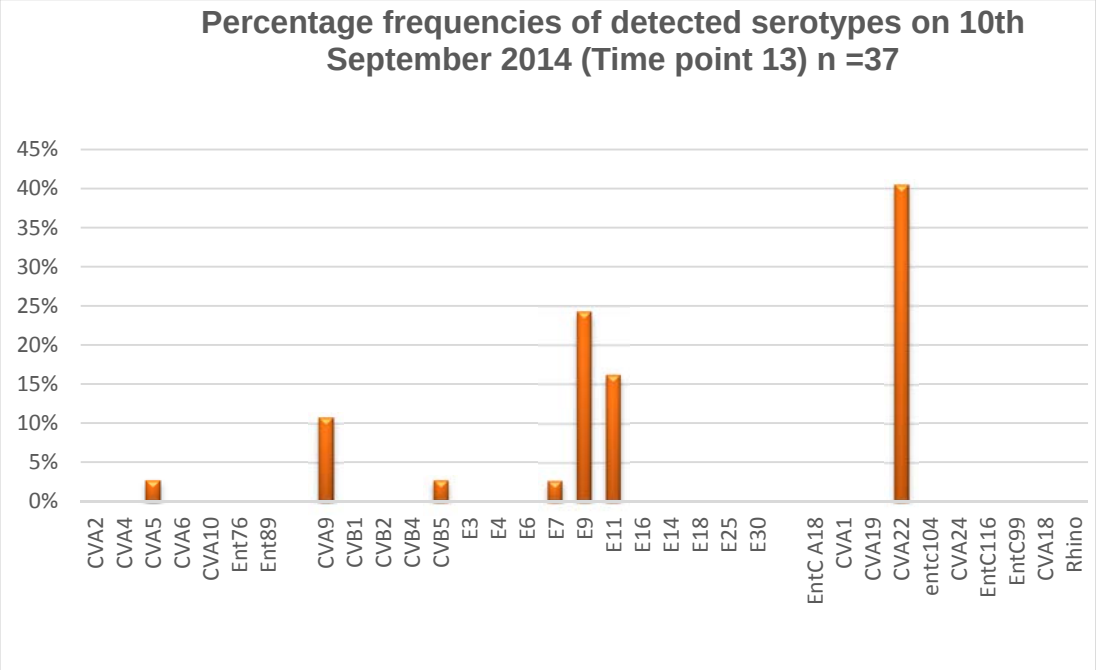


Figure 3.1m; Percentage frequencies of identified serotypes at time point 13

3.2.1c. Assessment of Results: *Coxsackievirus A22,* an *Enterovirus C* serotype was identified in all the samples except in the time point 11. It is however difficult to state definitively that it was the dominant serotype since over 50% of assay 1 serotypes identified were *Coxsackievirus A22* (CVA22). *Enterovirus C104,* a rare serotype was identified in sewage collected on the 4th and 18th of June 2014 (Figures 3.2.1f and 3.2.1g). *Enterovirus C104* has been associated with some cases of respiratory tract infections in Italy, Japan, Switzerland and China (Xiang *et al*., 2014). The fact that it was only identified in early summer when respiratory tract infections usually peaks is an interesting coincidence

The fewest number of serotypes were identified on the 23rd of April 2014. This is possibly due to PCR errors as all the colonies from assay 3 were identified as CVB5.

4.76% of the sequences detected in from sewage on the 26th of March 2014 were identified as CVA6. (Refer to Figure 3.2.1a). Coincidentally, CVA6 was identified in all typed cases of hand foot and mouth disease in Edinburgh between January and February 2014 (Sinclair *et al*., 2014). Identification of this virus in sewage underscores the fact that enteroviruses circulating in sewage are a marker for their prevalence in a particular community (Zheng *et al.*, 2013).

Some of the serotypes detected in sewage have been implicated in several disease manifestations. *Echovirus 30* for instance was identified to be the causative agent of aseptic meningitis outbreak in some parts of Finland particularly between August 2009 and September 2010 (Österback *et al*., 2015). Also, *Echovirus 11* a largely asymptomatic virus can cause a wide variety of clinical conditions like gastroenteritis, meningitis and encephalitis (Rubinstein *et al.,* 2000). Furthermore strains of both CVB5 and CVA10 have been associated in several outbreaks of hand foot and mouth disease in parts of South East Asia (Lu *et al.,* 2012; Hu *et al*., 2012).

CHAPTER 4 – DISCUSSION

4.1. Project aim

The aim of this study was to identify the types of enteroviruses circulating in Edinburgh at different time points through sewage screening, assess their temporal patterns and find out whether the detected serotypes were clinically significant. To do this, three RT-PCR assays were performed to amplify the VP4 and partial VP2 regions of enteroviruses after which the PCR products were cloned, sequenced and identified by online BLAST and phylogenetic analysis.

4.2. Background

Enteroviruses are quite diverse and many of their serotypes can infect human beings. Diagnosis at hospitals provides some insight into the types that are circulating in a particular area. However, many infections are subclinical and therefore an attempt to find the types of enteroviruses circulating in the city will not be very comprehensive if clinical diagnosis is the only way of studying enterovirus circulation. This study shows that, regular screening of sewage can give comprehensive information about the types of enteroviruses circulating in the city. Several studies monitoring community sewage for enteroviruses has been demonstrated in different parts of the world. In Wisconsin, USA, clinical isolates were compared with sewage isolates between August 1994 and December 2002 and it showed that the most commonly detected serotypes in sewage were similar to the most commonly detected serotypes in clinical specimens (Sedmak *et al.*, 2003). A study as well by Harvala *et al* also found similar pattern except that *Enterovirus C* serotypes were diverse in sewage but absent in in clinical specimens (Harvala *et al.,* 2013). The detection and characterisation of enteroviruses in sewage is an additional resource to enterovirus

surveillance and can help explain enterovirus associated disease trends and warn of possible future outbreaks in the area

4.1. Diversity in sewage:

In the attempt to identify the types of enteroviruses in Edinburgh, the study revealed that there was a high degree of serotype diversity circulating in local sewage. A total of *Enterovirus* 31 serotypes identified. The least diverse time point was on the 23rd of April 2014 (Time point 3) when only six serotypes *Enterovirus species* were identified. It is likely though that this result was due to PCR issues and not really a reflection on what serotypes were actually present. At this time point, 12 out of 20 sequenced colonies identified as CVA22 in assay 1 whereas all 20 colonies in assay 3 identified as CVB5. PCR picking up only one serotype is one assay was one of the major limitations of typing by nested PCR and cloning. Similar problems were encountered at time points 11, 12 and 13 where one assay generated several sequences of the same serotype. Time point 6 (4th June 2014) on the other hand exhibited the highest level of diversity with a total of 16 serotypes detected. *Enterovirus C104* a rare serotype was also identified at this time point.

4.2. Pattern.

Assessing the temporal pattern was one of the aims of this thesis. Due to the limited duration of the studies, a definitive long term temporal pattern could not be observed. All the three assays targeted the same region for amplification and cloning. However, despite the similarities in sensitivity in the detection limit assays, the data they generated on the same sewage samples were entirely different. This was probably due to preferential binding of the different antisense primers used in the various assays.

For example, no *Enterovirus A* serotypes were identified in assay 1. In assay 2 on the other hand, serotypes of *Enterovirus A* were only identified in 4 out of 13 time points compared to 8 out of 13 time points in assay 3. In merging these three assays, detected serotypes don't appear to follow any particular pattern with respect to the season that the sewage samples were collected. *Enterovirus B* however had more diversity in all the time points. At time point 11, there were no *Enterovirus C* serotypes identified which a bit surprising. *Enterovirus A* serotypes were also not detected at time point 4.

Enterovirus C104 was only identified twice on the 4th and 18th of June 2014. This may be significant as it is associated with respiratory tract infections was only detected twice in early summer when respiratory tract infections are quite high. No poliovirus strain was identified

4.3. Clinical significance

There was no concurrent diagnostic clinical data showing the types of enteroviruses circulating in the city during the time of study (March 2014 to September 2014). There has however been previous studies comparing *Enterovirus* species isolated in sewage with diagnostic clinical samples for *Enterovirus* surveillance in Scotland (Harvala *et al*., 2013).

16 serotypes belonging to *Enterovirus B* were detected in sewage during the course of the study and the most common serotypes were *Coxsackievirus B5* (CVB5: 83/598), E6 (9/598), E9 (22/598), E11 (44/598), CVA9 (37/598) and E30 (30/598). Comparing that to the diagnostic clinical data from published in in an article by Harvala *et al,* it appears that the six most common *Enterovirus* serotypes identified in

cerebrospinal fluid specimen collected in Edinburgh were E9, CVA9, CVB5, E6, E11 and E30, just like the isolates extracted from sewage during the sewage screen. No outbreaks however occurred during the period of study despite the fact that these serotypes have often been associated with large outbreaks due to appearance of new recombinant forms (Harvala *et al.*, 2013).

For species A, a total of 7 serotypes belonging to species A were detected during the sewage screen (CVA10, CVA6, CVA5. CVA2, *Enterovirus 76* and *Enterovirus 89*). On the other hand, the serotypes identified in the cerebrospinal fluid of young children with sepsis like illnesses included CVA2, CVA4, CVA6, CVA10, CVA16 and Enterovirus 71). The absence of CVA16 and *Enterovirus 71* in sewage may be due to temporal changes in the circulation of virus in Edinburgh. CVA16 and *Enterovirus 71* specimen were isolated between 2005 and 2010 whereas the sewage sample collection for this study only began in March 2014 (Harvala *et al.*, 2013).

Finally, *Enterovirus C* serotypes were detected in all but one of the time points that sewage was collected and a total of 8 serotypes were detected. However, no *Enterovirus C* was detected in the clinical specimens collected from 2004 to 2012. A lower pathogenicity of non-polio *Enterovirus C* strains may be the reason for this dichotomy (Melnick, 2007; Harvala *et al.*, 2013).

4.5 Recommendations/ Future Work

Through this project, the serotypes of enteroviruses circulating in Edinburgh have been identified and documented over a 6 months period using RT-PCR and gene

cloning. However, this opens up more possibilities to make enterovirus monitoring more efficient.

First of all, a deep sequence is probably a better way of finding the types of enterovirus present rather than cloning and sequencing of a select number of colonies. There are several *Enterovirus* serotypes that infect human beings and to select about 30 colonies after cloning is not likely to give a comprehensive overview of all the *Enterovirus* types in the sewage sample.

Deep sequencing may be a more appropriate technique to if the intent is to find all the types of enterovirus present. Typing based on VP4 and partial VP2 is not sufficient for identifying new species. This is because, only a significant divergence from a reference sequence in the VP1 region can (25% nucleotide or 12% amino acid divergence) result in assignment of a new serotype. Since designing primers to amplify the VP1 genomic region for all enteroviruses is challenging, a deep sequencing reaction will be far more efficient and faster.

To understand the temporal pattern, it would also be a good idea to find out the evolutionary changes that occur in the strains over a longer period of time. The nature of this project was time constrained and the significant evolutionary changes could not occur in that short period

Finally, concurrent data showing clinical manifestations of enteroviruses in Edinburgh during the same time period as the sewage screening would be helpful in assessing whether there is a correlation between sewage isolates and clinical presentation of enterovirus associated diseases.

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